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"There is one thing even more vital to science than intelligent methods;

and that is,

the sincere desire to find out the truth, whatever it may be."

C. Peirce, Collected Papers of C.S. Peirce (1935)

Opgedragen aan mijn ouders

Voor Gertie en "Jopie"

Contents

Abbreviations

BMT	:	Bone marrow transplantation
CD	:	Cluster designation
CyA	:	Cyclosporine A
CyA-AI	:	Cyclosporine A-induced autoimmunity
GVHD	:	Graft-versus-host disease
GVHR	:	Graft-versus-host reaction
Gy	:	Gray (= 100 Rad)
MAb	:	Monoclonal antibody
MHC	:	Major histocompatibility complex
SPF	:	Specified pathogen free
SS	:	Systemic sclerosis
TCR	:	T cell receptor

Introduction

This thesis deals with an experimental animal model of immune disease termed "syngeneic graft-versus-host-like disease" (sGVHD), also referred to as "Cyclosporine A-induced autoimmunity (CyA-AI)" or "bone marrow transplantation-associated immune disease" (BMT-ID). Its relevance to human disease is threefold. First, it is one of the few animal models that generates cutaneous lesions indistinguishable from those of scleroderma in man; this is a disease of obscure but undoubtedly immunological origin. Next, effector cells operative in this model have been advocated and used to treat hematopoietic malignancies, specifically non-Hodgkin's lymphomas, in man. Finally, when the experimental protocol designed in the rat is applied to man, identical autoimmune phenomena are observed. In the LEW rat the disease is brought about by subjecting the animal to lethal X-irradiation, followed by a syngeneic bone marrow transplantation, and transient administration of the immunosuppressive drug Cyclosporine A. The X-irradiation almost destroys the thymus and eradicates the peripheral T cell compartment. Next, pre-T cells derived from the bone marrow inoculum home into the thymus in order to recapitulate T cell ontogeny and repopulation of the periphery eventually, resulting in immunologically normal animals. However, this thymic and peripheral reprogramming is interfered with by Cyclosporine A, resulting in autoimmune disease.

Chapter 1, which is a literature review, deals with Cyclosporine A-induced autoimmune disease in various mammals and discusses the various mechanisms inclusive the thymus and peripheral effector cells by which autoimmune disease is brought about. The other chapters deal with own work.

For chapter 2, the question was asked whether or not Cyclosporine A-induced autoimmune disease was specific for the LEW rat strain; LEW rats are susceptible to this disease as well as for other cell-mediated autoimmune diseases like experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis to which the BN rat is resistant. In contrast, BN rats are susceptible to some antibody-mediated autoimmune diseases (e.g. mercury chloride-induced glomerulonephritis) to which LEW rats are resistant. Although both cell-mediated and antibody-mediated autoimmune reactions are elicited in CyA-AI, LEW rats were shown to be susceptible whereas BN rats were resistant.

As pointed out above the model of CyA-AI has been used in man to treat non-Hodgkin's lymphoma. Paradoxically, rats with CyA-AI and chronic dermatitis or scleroderma developed malignant tumors in the chronic phase of the disease. This

issue was examined in chapter 3. Indeed, LEW rats with chronic CyA-AI may develop a high incidence of sarcomas and adenocarcinomas. These tumors were analyzed histopathologically, immunohistochemically as well as biologically. The pathogenesis of these tumors is discussed.

In chapter 4 we have addressed the issue whether the chronic (cutaneous) scleroderma-like lesions in rats with CyA-AI are associated with autoantibodies to nuclear and/or cytoplasmic proteins. In man, autoantibodies to certain nuclear and/or cytoplasmic antigens are seen in up to 95% of the patients with systemic sclerosis.

In chapter 5 and 6 the role of the thymus and its putative effector cells in the model of CyA-AI is examined. In chapter 5 we show that the generation of autoreactive T cells - which cause CyA-AI - occurs during Cyclosporine A administration but remain "dormant" due to the immunosuppressive properties of Cyclosporine A. In chapter 6 finally we show that such cells exit from the thymus during recapitulation of T cell ontogeny that is already within 12 days after X-irradiation and during Cyclosporine A administration.

Chapter 1

Cyclosporine A-induced autoimmunity; An overview of the literature

Cyclosporine A-induced autoimmunity; An overview of the literature

Introduction

Cyclosporine A (CyA) is a drug with potent immunosuppressive activity which has been used extensively to prevent allograft rejection (White and Calne, 1982; Shevach, 1985; Hess *et al.*, 1986), to suppress graft-versus-host disease (GVHD) secondary to allogeneic bone marrow transplantation (BMT)(Tutschka *et al.*, 1979; Bacigalupo *et al.*, 1990), and in the treatment of several autoimmune diseases (Stiller *et al.*, 1984; Bolton *et al.*, 1982; Von Graffenried *et al.*, 1989). Paradoxically, under certain circumstances CyA may induce specific forms of autoimmunity. At least three experimental models of CyA-induced autoimmune disease have been described: syngeneic or autologous GVHD which occurs after X-irradiation, and syngeneic or autologous bone marrow transplantation provided CyA is given for a period of time after transplantation (Glazier *et al.*, 1983; Jones *et al.* 1989); organ-specific autoimmune disease in mice which occurs in neonatal mice treated with CyA (Sakaguchi and Sakaguchi, 1989); and organ-specific autoimmune disease in adult athymic mice after engraftment of a CyA-treated thymus (Sakaguchi and Sakaguchi, 1988)(Fig. 1.1). In this overview, mainly the model first mentioned will be dealt with.

Reconstitution of lethally irradiated rodents with autologous or syngeneic bone marrow usually leads to rapid recovery of such animals. A course of administration of the immunosuppressant CyA given for the first 6 weeks during this period of recuperation apparently does not interfere with recovery but elicits, surprisingly,

a T cell-mediated autoimmune disease which develops about 2 weeks after discontinuation of the drug (Glazier *et al.*, 1983). This CyA-induced autoimmune syndrome was originally termed syngeneic GVHD (Glazier *et al.*, 1983) and has also been referred to as CyA-induced autoimmune disease (CyA-AI)(Sorokin *et al.*, 1986) or BMT-associated immune disease (BMT-ID)(Bos *et al.*, 1990). The disease has been elicited in certain rat strains (Glazier *et al.*, 1983; Tutschka *et al.*, 1987; Geller *et al.*, 1989) and mouse strains (Cheney and Sprent, 1985; Bryson *et al.*, 1989) and may also occur in humans (Jones *et al.*, 1989).

The thymus has been demonstrated to play a pivotal role in this phenomenon (Sorokin *et al.*, 1986). Most likely, under CyA treatment recovery of thymic medullary stromal cells from the initial irradiation damage is delayed (Beschorner *et al.*, 1987b, 1987c). Probably due to this damage, the thymus fails to delete (or to negatively select) potentially autoreactive T cells which are also in untreated animals likely to develop in the generation of the (unselected) repertoire (Cheney and Sprent, 1985; Hess *et al.*, 1985; Hess and Fischer, 1989). Moreover, regulatory circuits which are able to suppress autoaggressive T cells are eliminated as well by the irradiation and apparently fail to recover in due time (Fischer *et al.*, 1989a). Consequently, symptoms develop which mimic the acute GVHD known to occur after transplantation of allogeneic bone marrow to lethally irradiated rats (Glazier *et al.*, 1983b). The model of CyA-AI offers new possibilities to investigate the role of the thymus in T cell development and challenges established concepts about the mechanisms eliciting GVHD.

The concept of CyA-induced syngeneic or autologous autoimmune disease was received with great scepticism (Chow *et al.*, 1988). GVHD is a disease that may develop as a consequence of a graft-versus host reaction (GVHR) upon transplantation across histoincompatibility barriers as postulated by Billingham (1967-1968), but similar pathology has been described in syngeneic or autologous CyA-AI (Glazier *et al.*, 1983b; Jones *et al.*, 1989; Hess *et al.*, 1992). From an immunological point of view, however, a GVHR could not occur in CyA-AI because of a lack of major or minor histoincompatibility differences between donor and recipient as demonstrated in the variant of the model using autologous bone marrow for reconstitution (Hood *et al.*, 1987; Hess *et al.*, 1992). So, other mechanisms must account for the induction of the pathology that is indistinguishable from the classical GVHD.

The precise mechanisms by which CyA induces autoimmune disease are not clear. Presumably, CyA affects immune tolerance by interfering with clonal deletion/anergy in the thymus, and the regeneration of the peripheral autoregulatory system which was eradicated by X-irradiation.

General features of CyA-AI associated pathology

CyA-AI has been originally described in the rat. Young female LEW rats which are lethally irradiated, reconstituted with syngeneic or autologous bone marrow and given a course of CyA over a period of about 4-6 weeks almost consistently develop CyA-AI about 2 weeks after discontinuation of the drug (Glazier *et al.*, 1983b; Sorokin *et al.*, 1986; Fischer *et al.*, 1989a; Fischer and Hess, 1990) (Fig. 1.1A). These rats show symptoms reminiscent of those seen in acute graft-versus-host disease: erythroderma of the extremities, a raised fur, a hunched appearance and sometimes diarrhoea (Glazier *et al.*, 1983b; Beschorner *et al.*, 1988c). Soon thereafter they suffer substantial weight loss and lose hair. Provided the animal does not die during this acute phase of CyA-AI, alopecia may progress to yield an almost entirely nude rat (Bos *et al.*, 1989d; Majoor, 1989). Survival of the acute phase of CyA-AI may be influenced by the microbiological status of the animals; i.e. SPF rats are less likely to succumb than conventionally housed animals (Sorokin *et al.*, 1986; Bos *et al.*, 1989d).

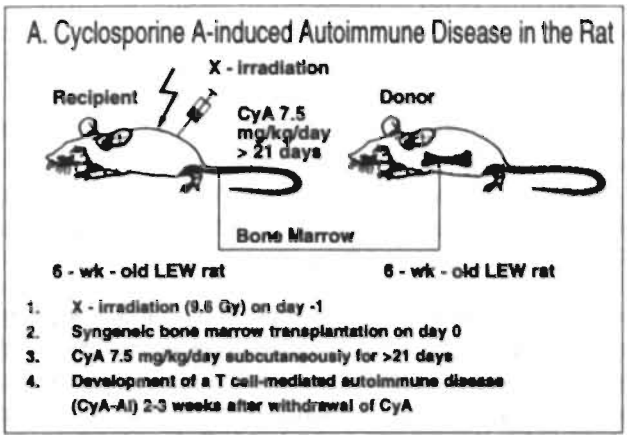
Histological abnormalities have been reported for many organs in the acute phase of the disease and include the skin, tongue, thymus, liver, intestine, salivary glands, esophagus, spleen, lymph nodes and the kidney (Glazier *et al.*, 1983a; Beschorner *et al.*, 1988c). In non-lymphoid organs, the abnormalities are confined to cellular infiltration; lymphoid organs show lymphoid atrophy (Glazier *et al.*, 1983a). In the later phase of CyA-AI the histological abnormalities appear to be restricted to the skin, tongue and thymus (Bos *et al.*, 1989d).

CyA-induced organ-specific autoimmunity

T cells play a pivotal role in generating various organ-specific autoimmune diseases in humans and animals (Schwartz and Datta, 1989). Hence, a critical issue in elucidating the pathogenetic mechanism of organ-specific autoimmune disease would be to determine how the thymic production of autoreactive T cells and their peripheral expansion/activation are controlled by the normal immune system, and which conditions are required for such self-reactive T cells to become active and elicit autoimmune disease.

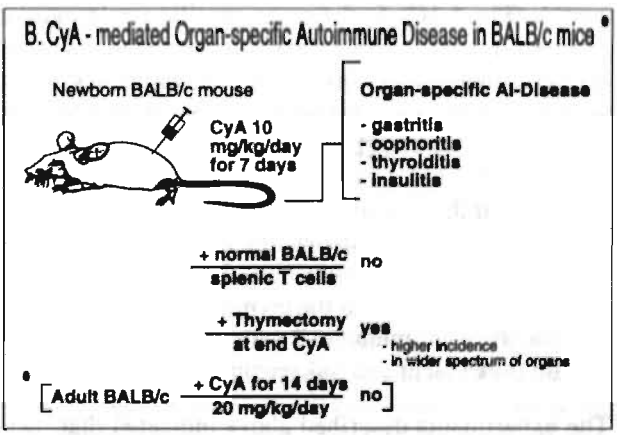
An altogether different CyA-induced autoimmune syndrome has been described in mice by Sakaguchi *et al.* (Sakaguchi and Sakaguchi, 1988, 1989). When CyA was administered to newborn BALB/c mice for 1 week from the day of birth, the mice subsequently developed organ-specific autoimmune diseases including gastritis, oophoritis, thyroiditis, and insulitis 3 months after discontinuation of CyA (Fig. 1.1B). This autoimmune pathology was accompanied by the appearance of serum autoantibodies specific for gastric parietal cells, oocyte, thyroglobulins, and cell surface antigens of the Langerhans islet cells. It was demonstrated for this model that CyA caused a reduction of $CD4^+CD8^-$ and

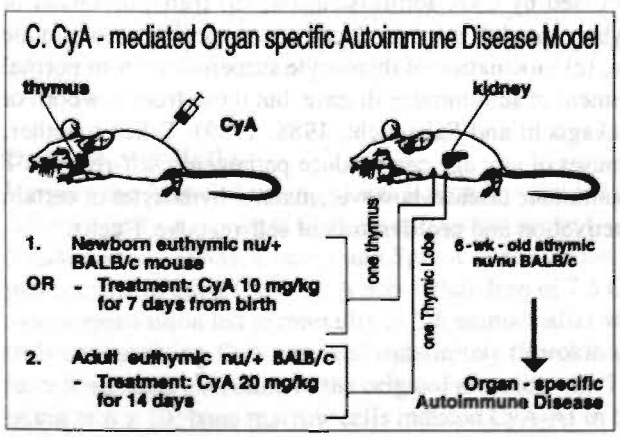
Figure 1.1: Experimental models of CyA-induced autoimmune disease.



A. Model of CyA-AI in the rat; 6 weeks old LEW are lethally X-irradiated (9.6 Gy), reconstituted with syngeneic bone marrow, and given 7.5 mg/kg/day CyA for 6 weeks (at least 3 weeks). A T cell-mediated autoimmune disease develops about 2-3 weeks after withdrawal of CyA.

B. Model of CyA-mediated organ-specific autoimmune disease in BALB/c mice; newborn mice are treated with 10 mg/kg/day CyA for 7 days. Organ-specific autoimmune disease including gastritis, oophoritis, thyroiditis and insulitis was histologically confirmed about 3 months after CyA discontinuation. Adult BALB/c mice treated with 20 mg/kg/day CyA did not develop organ-specific autoimmune disease.





C. Model of CyA-mediated organ-specific autoimmune disease in athymic BALB/c mice after engraftment of a CyA-treated thymus from euthymic mice. Six weeks old athymic nu/nu BALB/c mice were engrafted with either one thymus from euthymic nu/+ BALB/c mice treated with 10 mg/kg/day CyA for 7 days or one thymic lobe from adult euthymic nu/+ BALB/c mice treated with 20 mg/kg/day CyA for 14 days, under the renal capsule. Organ-specific autoimmune disease develops as outlined in fig. 1.1B

$CD4^-CD8^+$ single-positive mature thymocytes and peripheral lymphoid atrophy. The same dose and period of CyA administration starting from 1 week after birth was less effective, and CyA administration to adult mice failed to induce organ-specific autoimmune disease. Autoimmune disease was preventable by inoculation of normal splenic T cells immediately after neonatal administration of CyA. On the other hand, thymectomy immediately following withdrawal of neonatal CyA treatment produced a higher incidence of organ-specific autoimmune disease, as well as disease in a wider spectrum of organs. This indicates that removal of the thymus immediately after neonatal CyA-treatment may permanently sustain a deficiency of suppressor T cells in the immune system, allowing a variety of self-reactive T cells to expand more easily and cause autoimmune disease in a wider spectrum of organs (Sakaguchi and Sakaguchi, 1989) (Fig. 1.1B).

Assuming that self-reactive T cells can be controlled in the periphery by a T cell-dependent down-regulation, autoimmune disease may develop when pathogenic self-reactive T cells are released from the thymus to the T cell-deficient or -eliminated periphery. Thus, organ-specific autoimmune diseases developed also in athymic nude (*nu/nu*) mice after engraftment of the thymus from heterozygous *nu/+* mice treated with CyA under the renal capsule (Fig. 1.1C). In the organ-specific autoimmune diseases, T cells produced by and released from the thymus grafts appeared to exert antigen-specific help on the host-derived autoantibody-forming B cells and/or conduct cell-mediated immune reactions towards specific self-antigens. Development of autoimmune disease in the *nu/nu* mice was prevented by co-inoculation of thymocyte suspensions from normal *nu/+* mice, but not from CyA-treated *nu/+* mice. Co-transplantation of normal *nu/+* mouse thymus with CyA-treated thymus also prevented development of autoimmune disease. Inoculation of spleen cells from normal adult *nu/+* mice prevented autoimmune disease, but inoculation of those from newborn *nu/+* mice did not. This indicates once more that CyA appears to interfere selectively with the thymic production of certain suppressor T cells controlling self-reactive (autoimmune) T cells, allowing the latter to expand and cause autoimmune disease (Sakaguchi and Sakaguchi, 1988).

The experiments described above indicated that: (a) development of mature single-positive thymocytes was suppressed by CyA administration; (b) transplantation of thymuses from CyA-treated newborn or adult mice produced organ-specific autoimmune disease in syngeneic *nu/nu* mice; (c) inoculation of thymocyte suspensions from normal adult mice could inhibit development of autoimmune disease, but those from newborn or CyA-treated mice could not (Sakaguchi and Sakaguchi, 1988, 1989). Taken together, these findings suggest that thymuses of any age can produce pathogenic self-reactive T cells eliciting organ-specific autoimmune disease, however, mature thymocytes or certain T cells may inhibit peripheral activation and proliferation of self-reactive T cells.

Conditions for induction of CyA-AI

Thymic involvement

The general features of CyA-AI are suggestive of an autoimmune phenomenon which could at least in part be due to a perturbation of the normal induction of tolerance for 'self' in the thymus. Several studies, using different approaches, have clearly demonstrated an essential role of the thymus in bringing about CyA-AI. Shielding of the thymus from irradiation prevented the development of CyA-AI in recipients further subjected to the usual protocol of BMT and administration of CyA (Glazier *et al.*, 1983a). Furthermore, CyA-AI could not be elicited in adult rats thymectomized prior to X-irradiation and BMT. This failure to develop CyA-AI could not be restored by transfer of various kinds of lymphoid cells (including thymocytes), but was restored after grafting of intact thymic lobes in the axilla prior to X-irradiation and CyA treatment (Sorokin *et al.*, 1986). Further evidence for the essential role of the thymus in CyA-AI was derived from a somewhat different mouse model. Female newborn or adult *nu/+* mice were given CyA for 1 or 2 weeks respectively and next their thymuses were grafted to adult *nu/nu* recipients. In this model, extensive pathology was seen to develop including gastritis, oophoritis, thyroiditis and insulitis (Sakaguchi and Sakaguchi, 1988). The evidence from the studies cited above unequivocally established that the thymus is pivotal in the establishment of CyA-AI. The data from the mouse model further suggest that irradiation as applied in the rat model may serve another purpose than just contributing to the disturbance of correct education of T cells in the thymus. Apparently, irradiation also contributes to the elimination of a (peripheral) counteractive autoregulatory mechanism. On the other hand, the observation that simply shielding the thymus from irradiation prevents CyA-AI (Glazier *et al.*, 1983a) proves that the combination of irradiation and CyA administration is required to perturbate regular tolerance induction in the thymus. In contrast, Beschorner *et al.* (1991) have reported acute CyA-AI but not chronic-type CyA-AI in non-irradiated rats treated with CyA for 32 days and thymectomized immediately after discontinuation of CyA. In their view, self-reactive effector lymphocytes migrated to the periphery during CyA treatment. However, thymectomy would prevent development of counteractive regulatory T cells and thus allow expansion of autoreactive T cells and consequently tissue damage.

Dose of irradiation

Varying the dose and timing of irradiation has yielded additional information about its effects. Firstly, a course of CyA alone does not evoke CyA-AI in a non-irradiated animal (Glazier *et al.*, 1983a; Cheney and Sprent 1985). Secondly, lethal irradiation may be no prerequisite to elicit CyA-AI. A non-lethal dose of 7.5 Gy without additional bone marrow reconstitution led to mortality in 5/6 animals after withdrawal of CyA, whereas controls not receiving CyA survived indefinitely (Sorokin *et al.*, 1986). In contrast, others have found that execution of the original protocol with 7.5 Gy irradiation and transplantation of 6×10^7 bone marrow cells induced CyA-AI in only one-third of the rats; a dose of 5.0 Gy or lower was completely ineffective (Fischer *et al.*, 1989a).

Dose of CyA and timespan of administration

The dose of CyA needed to elicit CyA-AI does not seem to be very critical. Originally, a dose of 7.5 mg/kg/day for 40 days was reported to suffice, although the same dose for 20 days was only effective in half of the recipients (Glazier *et al.*, 1983a, 1983b). However, the same group of investigators subsequently used 15 mg/kg/day for 40 (Hess *et al.*, 1985) or 30 days (Fischer *et al.*, 1989a). Recent data showed that 5 mg/kg/day is still effective provided donors and recipients of one month of age were used. A dose of 1 mg/kg/day failed to induce CyA-AI (Fischer and Hess, 1990). Extension of the period of treatment to 80 days had neither effect on the kinetics of development of CyA-AI nor on its severity (Bos *et al.*, 1988).

Influence of species and age

The rat model as originally described proved to be easily reproducible using young female LEW ($RT1^l$) rats; males and females are equally susceptible. In two other studies, female Louvain (Lou/M; $RT1^u$) and (LEWxBN)F1 rats were shown to be susceptible as well (Tutschka *et al.*, 1987; Geller *et al.*, 1989). On the other hand, the Brown Norway (BN; $RT1^n$) rat strain is (relatively) resistant to the induction of CyA-AI (Wodzig *et al.*,1993 this thesis chapter 2). Susceptibility of LEW and (LEWxBN)F1 rats, taken together with the resistance of BN rats, suggests that susceptibility is genetically a dominant trait.

More discussion has been raised about the reproducibility of the model in mice. Originally, Cheney and Sprent (1985) described CyA-AI in C57Bl/6 or (C57Bl/6 x CBA/J)F1 mice after administration of 10 mg CyA/kg/day for 5-6 weeks. Next, it was reported, however, that CyA-AI could not be elicited in C57Bl/6BOM mice by oral administration of even 50 or 100 mg/kg/day for 40 days after lethal irradiation and syngeneic BMT (Chow *et al.*, 1988). This study was confirmed in CBA/J mice given 25 or 50 mg CyA/kg/day subcutaneously (Parfrey and Prud'homme, 1990). In contrast, induction of CyA-AI in DBA/2 and Balb/c mice was demonstrated recently using a dose of 15 mg CyA/kg/day and, probably most importantly, using mice aged 3-4 weeks instead of mice 6-8 weeks of age like used in the studies that failed to demonstrate CyA-AI (Bryson *et al.*, 1989). Indeed, also in the rat very young animals (4-6 weeks) develop CyA-AI more readily than animals about 12 weeks of age (Beschorner *et al.*, 1988d; Fischer and Hess, 1990). Therefore, the debate about a different sensitivity of rats and mice to the induction of CyA-AI can probably be ascribed apart from the strains used to the use of too old mice in the studies that failed to demonstrate CyA-AI.

Although the occurrence of CyA-AI has not yet been demonstrated in other species than rats, mice, and humans, at the level of the thymus effects comparable to those observed in rodents (see below) have been demonstrated in chickens (Bucy *et al.*, 1990).

Adoptive transfer of CyA-AI

The protocol as described above for rodents apparently elicits (or fails to suppress) an autoimmune effector mechanism(s) which may cause severe disease. The similarity of the lesions in CyA-AI with those occurring in GVH disease and the essential role of the thymus suggested involvement of (autoaggressive) T cells. Hence, already the first paper on

CyA-AI described investigations on the possibility to transfer the disease to secondary, syngeneic recipients. It proved impossible to transfer the disease to untreated rats; irradiation (4.2 or 10.2 Gy) and bone marrow reconstitution of the recipients was required in order to adoptively transfer CyA-AI (Glazier *et al.*, 1983a, 1983b). Later, the issue of preparation of prospective recipients of CyA-AI effector cells was investigated further by Fischer *et al.* (1989). In this study, irradiation of the secondary recipient with 7.5 Gy sufficed for 100% successful adoptive transfer of the disease; 5.0 Gy yielded success in only 1 out of 7 recipients. Interesting was the demonstration in the same study that Cyclophosphamide (100 or 200 mg/kg) was also effective in preparing the secondary recipients, whereas it was not so in the induction of CyA-AI. These data suggested that the lethal irradiation applied in the regular protocol for induction of CyA-AI in fact required two targets: one probably residing in the thymus and another one extrathymically.

Another issue raised was what kind of cells were able to transfer CyA-AI. The earliest study in rats demonstrated that spleen cells were effective, 10^8 cells being used for these transfers (Glazier *et al.*, 1983a, 1983b). In mice comparable results were obtained; this study demonstrated in addition the effector cells to be T lymphocytes (Cheney and Sprent, 1985). There is considerable debate concerning the potency of T lymphocytes subsets from CyA-AI rats in transferring disease to secondary recipients. Fischer *et al.* (1990) demonstrated the requirement for both splenic $CD4^+$ and $CD8^+$ subsets (both 7.5×10^6) to transfer CyA-AI effectively. Transfer of large numbers ($>3 \times 10^7$) of $CD8^+$ cells from animals with acute CyA-AI resulted in the induction of CyA-AI in the secondary recipients. However, lower numbers of $CD8^+$ cells could only transfer CyA-AI if accompanied by small numbers of $CD4^+$ T cells from donors with CyA-AI. The $CD4^+$ subset cells harvested during acute CyA-AI were ineffective by themselves. On the other hand, Sorokin *et al.* (1986) were able to transfer disease with as few as 3×10^5 $CD4^+$ lymph node cells from animals with active CyA-AI; B cells and $CD8^+$ cells were far less effective. These discrepancies may be due to the fact that *in vitro* activated $CD4^+$ cells may express the $CD8\alpha\alpha$-chains ($CD4^+CD8\alpha^+\beta^-$) to which the anti-CD8 antibody (OX8; used to identify the CD8 subset) is directed; hence, these studies should be repeated using a MAb directed to the CD8ß-chain which is not expressed on activated $CD4^+$ cells (Torres-Nagel *et al.*, 1992).

Also 8×10^7 or 1.8×10^8 thymocytes obtained 30 days after discontinuation of CyA from rats with CyA-AI have been successfully used in adoptive transfers to irradiated and bone marrow-reconstituted recipients. Pathology in these recipients at 3-5 weeks post-transfer was reminiscent of chronic graft-versus-host disease and involved the skin and the tongue. In these organs a marked preponderance of $CD4^+$ lymphocytes over $CD8^+$ lymphocytes was noted. In the thymus a moderate loss of epithelial cells was seen in the cortex and a near-total loss of epithelial cells in the medulla (Beschorner *et al.*, 1988a). Inspection of the thymus of irradiated but non-reconstituted rats at 4-48 hrs post-transfer revealed increased lymphocytic exocytosis of Hassall's corpuscles associated with $CD8^+$ lymphocytes and epithelial cell injury as compared to controls. These data were interpreted as indicative for the presence of autoreactive cells in the thymus of rats with CyA-AI that are able to damage thymic epithelial cells (Beschorner *et al.*, 1988b). As

described in this thesis, however, thymocytes harvested up to 14 days after discontinuation of CyA could not transfer the disease (this thesis chapter 6).

Another transfer model has been described by Cheney *et al.* (1985). In this study, spleen plus lymph node cells from non-irradiated mice given CyA for 5-6 weeks were obtained one day after withdrawal of CyA and transferred to irradiated syngeneic recipients. Acute, fatal CyA-AI developed in these mice but not after transfer of such cells to non-irradiated recipients. In our hands, transfer of 2.5 x 10^7 spleen and lymph node cells from non-irradiated, CyA treated rats failed to induce disease in secondary, irradiated recipients (Wodzig *et al.*, 1991). However, we harvested the cells 2 weeks after discontinuation of CyA. By that time, the putative effector cells may have shifted from the lymphoid organs to the peripheral tissues and therefore be no longer included in sufficient amounts in the cell population transferred.

The adoptive transfer systems described above also opened the possibility to analyze the cells that were likely to be involved in the suppression of CyA-AI effector cells in non-irradiated or non-Cyclophosphamide treated rats. The first attempts to suppress the effector cells in adoptive transfer experiments by addition of an equivalent amount of spleen cells from normal rats were unsuccessful, as were experiments in which normal spleen cells or intraperitoneal thymus grafts were given simultaneous with the reconstituting bone marrow cells, thus prior to CyA treatment (Glazier *et al.*, 1983a, 1983b). However, by changing the ratio of normal spleen cells to those from CyA-AI rats to 10:1 (Sorokin *et al.*, 1986) or 2:1 (Fischer *et al.*, 1989a, 1989b), suppression of CyA-AI could be demonstrated in adoptive transfers. Furthermore, it was shown that the regulatory component within the normal spleen cell population consisted of T cells (Fischer *et al.*, 1989a) and that both $CD4^+$ and $CD8^+$ T cells are needed in this regulatory population in order to be effective (Fischer *et al.*, 1989b; Hess *et al.*, 1989).

In the mouse model in which transplantation of *nu/+* thymus from CyA-treated mice into *nu/nu* recipients was performed a counteractive regulatory T cell system is obviously absent from the host. Also in this model the existence of a regulatory mechanism in phenotypically normal (*nu/+*) mice could be demonstrated. Inoculation of adult, normal *nu/+* thymocytes or spleen cells at the time of transplantation of a CyA-AI *nu/+* thymus prevented the development of CyA-AI, as did co-transplantation of a normal nu/+ thymus. Spleen cells from newborn *nu/+* mice were ineffective in this respect. These findings were interpreted to indicate permanent production of potentially autoaggressive T cells and their dominant regulatory counterparts in the thymus (Sakaguchi and Sakaguchi, 1988).

The nature of the effector cells that cause pathology in CyA-AI remains an enigma. Hess *et al.* (1985) have demonstrated that CyA-AI rats have $CD8^+$ cytotoxic T cells directed to public determinants on rat MHC class II molecules, including their own. In contrast, the data from adoptive transfer studies (Sorokin *et al.*, 1986), the coincidence of reappearance of $CD4^+$ T cells in the periphery and the development of symptoms of disease (Bos *et al.*, 1988, 1989a), and the prevalence of $CD4^+$ cells over $CD8^+$ cells in target tis-

sues (Beschorner *et al.*, 1988a), however, suggest that CD4⁺ T cells play an important role in bringing about the pathology in CyA-AI.

Current understanding of CyA-AI

Lethal or near-lethal irradiation of young rodents, followed by reconstitution with autologous bone marrow or marrow from young, syngeneic donors, and administration of CyA for about 5 weeks yield the disease 2-3 weeks after discontinuation of the drug. About 10 Gy irradiation and CyA are needed at the level of the thymus to "produce" the autoaggressive T cells; about 7.5 Gy (or treatment with the immunosuppressive drug Cyclophosphamide) suffices to remove a normally prevailing peripheral regulatory T cell circuit, which is obviously also eliminated by 10 Gy whole body irradiation (Fischer *et al.*, 1989a). This suppressor mechanism apparently does not regenerate concomitantly with the generation of the autoaggressive cells. The requirement to use young recipients may reflect different sensitivity of the thymic medulla of young and old rodents to the regimen for induction of CyA-AI (Beschorner *et al.*, 1987c, and see below). The use of bone marrow cells from young donors on the other hand is mandatory, since marrow from older donors may contain regulatory T cells and/or their precursors which may suppress CyA-AI in vivo (Fischer and Hess, 1990). Manifestation of pathology coincides with the reappearance of the peripheral CD4⁺ T cell subset (Bos *et al.*, 1988; Fischer *et al.*, 1991) but the effector mechanisms causing the pathology are not yet clear.

In the next part, we will review the composition of the thymus including its micro-environment.

The thymus

The mammalian thymus is a bilobal structure that lies on the pericardium, the aortic arch and its branches, and the trachea. Each lobe is surrounded by a collagenous connective tissue capsule (Kendall, 1988). At the histological level, three main thymic areas can be defined in each lobe: the subcapsular zone that lies just under the connective tissue capsule; the cortex, which forms the major outer area; and the centrally located medulla (Fig. 1.2). Each area is characterized by a distinctive lymphoid and stromal cell composition (Ritter and Crispe, 1992). At some places the connective tissue capsule intrudes deep into the tissue to the level of the cortico-medullary junction, thus creating septa that divide the organ into many pseudo-lobules. These septa carry both the vascular and neuronal supply to and from the thymus, while branches from the septa give rise to perivascular spaces within the thymus (Ritter and Crispe, 1992).

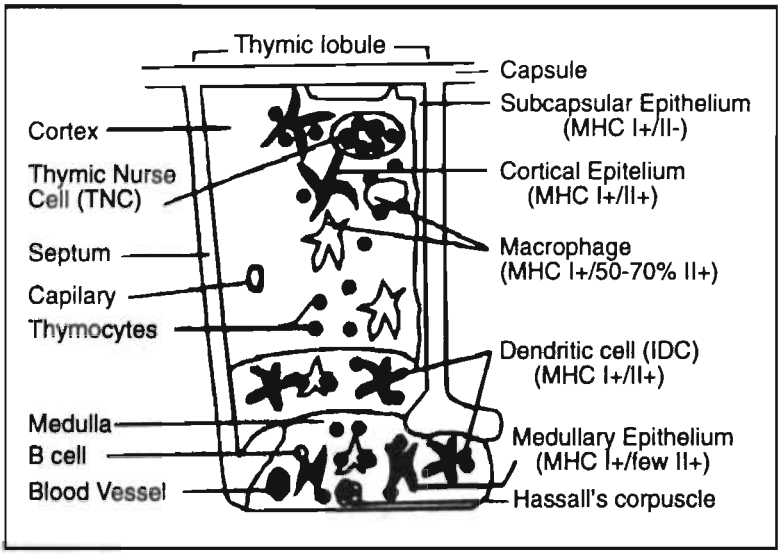


Figure 1.2: Schematic view of a thymic lobule and its microenvironment. *For explanation see text. Reproduced in part from Boyd et al., 1991.*

Cellular composition of the thymus

The most numerous cells in the thymus are (immature) lymphocytes which lie within a framework provided by several types of stromal cells. The major component of this stroma is the epithelium, which is molecularly and functionally heterogenous and provides the structure within which all the other cell types reside (Van Ewijk, 1988; Godfrey *et al.*, 1990).

Monoclonal antibody (MAb) studies have shown 5 major 'clusters of epithelial staining' (CTES) which fit well with morphologically defined epithelial populations within the thymus; expression of MHC antigen depends on the type and localization of epithelial cells. BM-derived macrophages and dendritic cells (interdigitating cells, IDC) form the other major stromal cell populations (Van Ewijk, 1988). Macrophages can be distinguished by differences in their endogenous enzymes, and by their antigenic phenotype defined by MAb's (Milicevic *et al.*, 1989). Additional cell types form minority populations within the thymus, e.g. myoid cells, B lymphocytes, neutro- and eosinophils (Ritter and Crispe, 1992).

Subcapsular region

Approximately 5% of thymic lymphoid cells reside in the subcapsular area; these are mainly large blast cells, with high mitotic activity. Epithelial cells in the subcapsular region form a layer one to two cells deep, with the outermost layer lying on the basement membrane. This type of epithelial cells stained with MAb's CTES I and II, expressed

MHC class I molecules at a high density, but MHC class II antigen expression was absent (Ritter and Crispe, 1992)(Table 1.1).

Table 1.1: (Sub)populations of thymic cell types.

Cell types	CTES MAb staining[a]	MHC class II expression[b]
Epithelial cells		
subcapsular	I, II	negative
cortical (incl. TNC)	I, III	positive
medullary	I, IIp, IV, Vp	few positive
- Hassall corpuscles	I, II, IV, V	negative
Dendritic cells		positive
Macrophages		
cortical		50-75% positive
cortico-medullary		50-75% positive
medullary		50-75% positive

[a]CTES, clusters of epithelial staining; p, only a subpopulation is positive. [b]All cell types expressed MHC class I antigen at a high density on their surface. (Reproduced in part from Ritter and Crispe, 1992).

Cortex

The majority of thymocytes (80-85%) are found in the cortex. Ninety to 95% of thymic lymphoid cells are eliminated during T cell receptor repertoire selection (Scollay *et al.*, 1980). Death occurs by apoptosis, a programmed cell death mechanism whereby endonucleases cleave nuclear DNA into smaller pieces (Wyllie *et al.*, 1984). Cortical thymocytes are mostly small, predominantly non-dividing cells that are closely packed together. Epithelial cells in the cortex (CTES I/III) have very long cytoplasmic processes, forming a network throughout this region of the thymus and expressing both MHC class I and II antigen on their surface (Ritter and Crispe, 1992). Some of these epithelial cells (Thymic Nurse Cells, TNC) appear to completely surround and enclose 10 to 250 lymphocytes (Van Vliet *et al.*, 1984). Macrophages are scattered throughout the cortex and are sometimes surrounded by a rosette of proliferating lymphocytes (Kyewski *et al.*, 1982). All macrophages expressed MHC class I molecules but only a subpopulation is MHC class II positive (Table 1.1). Fragments of apoptotic cells are predominantly found in cortical macrophages (Kendall, 1990).

Medulla

The remaining 10% of thymocytes are located in the medulla. These lymphocytes are very similar to mature peripheral T lymphocytes in size, phenotype, and functional maturity, and probably represent the end-product of intra-thymic T cell development. The epithelium of the medulla (CTES I, IIp, IV, V) consists of oval shaped cells with shorter, spatula-like processes that do not form connections with each other as close as

seen with the epithelial cells in the cortex (Ritter and Crispe, 1992). Medullary epithelial cells are MHC class I positive but only a subpopulation expresses MHC class II antigen on their surface. Concentric whorls of epithelial cells are also found in the medulla. These Hassall's corpuscles vary considerably in size according to the species and are MHC class II negative. Macrophages and dendritic cells are scattered throughout the medulla and both cell types are MHC class I positive. Dendritic cells and a subpopulation of macrophages (50-75%) expresses also MHC class II antigen on their cell surface (Table 1.1). Myoid cells (containing skeletal muscle-like striations) are found in clusters often closely associated with Hassall's corpuscles. B lymphocytes, many with an immature phenotype, are also found in the medulla (Pabst *et al.*, 1989).

Lymphocyte development

On entering the thymus from the blood stream, pro-thymocytes ($CD4^-CD8^-CD3^-$) are induced to undergo a program of proliferation, gene rearrangement, and differentiation under the influence of stromal cells types in various thymic microenvironments and resulting in the production of large numbers of immature double positive $CD4^+$ $CD8^+$ (TCRαß/CD3low) thymocytes expressing a diverse repertoire of T cell receptor (TCR) specificities (Jenkinson *et al.*, 1992). This repertoire is subject to stringent selection and the specificity of the αß TCR determines whether cells (1) die a programmed death when the TCR fails to bind to any ligand, (2) are immediately deleted when the TCR binds to self-MHC plus self-peptide (negative selection) or (3) are rescued when the TCR binds to self-MHC ligands in thymic cortical epithelium with foreign peptide (positive selection) (Von Boehmer, 1992). Cortical epithelial cells are involved in "positive selection" of MHC-restricted T cells, whereas interdigitating cells (IDC) and thymic medullary epithelial cells are involved in "negative selection" (Nikolic-Zugic, 1991). Positively selected cells (recognizing self-MHC molecules) are signaled to mature into single positive $CD4^+CD8^-$ (TCRαß/CD3hi) or $CD4^-CD8^+$ (TCRαß/CD3hi) T cells, depending on the specificity of their receptor, i.e. binding specificity for MHC class II-associated peptide or class I-associated peptide respectively (Von Boehmer, 1992).

In the next part, we will review the effects of CyA on the (irradiated) thymus and undertake an attempt to link those to the mechanisms purportedly operative in the CyA-AI model.

Effects of CyA on the non-irradiated thymus.

The effects of CyA on the thymus are most marked on the medulla; the cortex usually being only marginally affected. One of the earliest observations on the effect of CyA on the thymus was made in mice (Ryffel *et al.*, 1981). Within 4 days after oral administration of a bolus of 250 mg/kg CyA a marked reduction of thymic weight was recorded. It

is striking that in this study the observed effect was ascribed to a depletion of cortical thymocytes, whereas many papers published thereafter stressed the prominent and predominant effect of CyA on the thymic medulla. Perhaps stress, caused by the single, extremely high dose of CyA used in this study, and its oral administration accounted for this discrepant observation (Clarke and MacLennan, 1986). In favour of the influence of the route of administration is a study in rats in which CyA was administered orally as well at a dose of 100 mg/kg/2 days for 3 weeks (Thomson *et al.*, 1981). In this report, neither macroscopic or histological abnormalities, nor a significant effect on thymic weight was noticed as compared to controls. Only in the peripheral blood of the CyA-treated rats lymphopenia and the appearance of atypical lymphocytes was recorded. On the other hand, when normal LEW rats were given 7.5-100 mg CyA/kg/day subcutaneously a rapid depletion of the medullary thymocytes was observed (Schuurman *et al.*, 1990). After 50 mg CyA/kg/day for 2 weeks distinct medullary regions were almost absent from the thymus (Beschorner *et al.*, 1988d). Both in this study and in the last afore-mentioned the thymic architecture was restored within 3 weeks after withdrawal of CyA. The observation on the predominant effect of CyA on the thymic medulla was, however, confirmed in a study in which the oral route was used for CyA administration. After feeding rats a diet containing 0.015% CyA for 8-10 weeks thymic involution was evident. Cortical atrophy was only slight, but medullary atrophy was scored as moderate and in some rats even no remnants of the thymic medulla were detected at all (Demetris *et al.*, 1984). Thus, it appears that subcutaneous administration of CyA exerts stronger effects on the thymus than oral administration, although prolonged feeding of CyA may cause comparable damage, especially to the medulla.

Effect on medullary stromal cells

CyA has been shown to exert its main effect on the thymic medulla which is composed of lymphocytes, epithelial cells, macrophages, dendritic cells, and histiocytes. Although the medulla is considerably reduced in size due to CyA administration, representatives of all normal medullary components were still present. The CyA-induced damage of the medulla was reflected in decreased numbers of macrophages (Kanariou *et al.*, 1989), and loss of dendritic cells and histiocytes (Beschorner *et al.*, 1987b; Kanariou *et al.*, 1989). Probably at least in part associated with the loss of the above-mentioned cell types was a reduced expression of MHC class II molecules in the medulla (Beschorner *et al.*,1987a, 1987b, 1987c; Kanariou *et al.*, 1989; Schuurman *et al.*, 1990), a general decrease in (keratin$^+$) epithelial cells (Hattori *et al.*, 1987, Kanariou *et al.*, 1989), ablation of epithelium of Hassall's corpuscles (Beschorner *et al.*, 1987b, 1987c; Linder, 1987) and reduced MHC class I expression (Linder, 1987; Tanaka *et al.*, 1988; Majoor and Van Breda Vriesman, 1989).

Effect on cortical stromal cells

The thymic cortex of CyA-treated animals was morphologically indistinguishable from that of control animals although increased numbers of epithelioid cells containing pyknotic nuclear debris (Demetris *et al.*, 1984) and disorganization of the reticular tis-

sue (Fein *et al.*, 1989) have been reported. Although the distribution of cortical macrophages did not change significantly, their morphology and enzyme histochemistry altered markedly (Milicevic *et al.*, 1989).

Many of the above-cited reports stress the striking reversibility of the aberrations observed; usually within 2-3 weeks after withdrawal of CyA the thymus had regained its normal architecture.

Effects on T lymphocyte development

Already in early reports on the effects of CyA *in vivo* a peripheral lymphopenia was noticed which was associated with the appearance of atypical, blast-like lymphocytes (Thomson *et al.*, 1981). Possibly, these cells represented the peripheral lymphocyte population later identified as carrying both the CD4 and the CD8 T cell surface molecules ($CD4^+8^+$)(Godden *et al.*, 1985). According to this report, both CyA and a (vascularized heart) graft were necessary to generate these cells, which could constitute up to 40% of the peripheral leukocyte population. In the study of Thomson *et al.* (1981) sheep red blood cells, administered to evaluate the immunosuppressive effect of CyA, may have substituted for the role of the graft as proposed in the work of Godden *et al.* (1985).

Double positive $CD4^+8^+$ T cells have also been reported to be present in rats with CyA-AI (Hess *et al.*, 1987). As long as CyA is administered after lethal irradiation and marrow reconstitution the absolute and relative numbers of peripheral $CD4^+$ cells are markedly reduced, whereas $CD8^+$ cell numbers are less clearly affected (Hess *et al.*, 1987; Bos *et al.*, 1988; Fischer *et al.*, 1991). However, in the periphery of these rats (and of unirradiated, CyA-treated rats) 20-30% of the T cells were shown to have the $CD4^+CD8^+$ phenotype (Hess *et al.*, 1987). Most investigators consider the release of $CD4^+8^+$ thymocytes into the circulation unusual (Scollay *et al.*, 1984), although others have defended this export from the thymus to play a role in the clearing of $CD4^+8^+$ T cells with unwanted specificities from the body (Rothenberg, 1990). On the other hand, the failure of $CD4^+8^+$ cells to continue their normal development in the thymus in the presence of CyA has been firmly established. In rodents CyA treatment was shown to reduce the number of single positive $CD8^+$ and especially $CD4^+$ cells in the thymus (Beschorner *et al.*, 1987a; Heeg *et al.*, 1989; Hiramine *et al.*, 1988; Hiramine *et al.*, 1989; Kanariou *et al.*, 1989; Sakaguchi and Sakaguchi, 1989; Bucy *et al.*, 1990). These findings were corroborated by studies using mouse fetal thymus organ cultures, which demonstrated an almost complete inhibition of development of single positive $CD4^+$ and $CD8^+$ cells in the presence of CyA (Kosugi *et al.*, 1989b; Matsuhashi *et al.*, 1989; Takeuchi *et al.*, 1989, Siegel *et al.*, 1990). Furthermore, these in vitro studies demonstrated that the development of $CD3^+4^-8^-$ $TCR\alpha\beta^-$ ($TCR\gamma\delta^+$?) T cells was not interfered with by CyA (Kosugi *et al.*, 1989b; Matsuhashi *et al.*, 1989; Siegel *et al.*, 1990); that MHC class II^+ cells do not disappear under these culture conditions (Takeuchi *et al.*, 1989; Siegel *et al.*, 1990); and finally that IL-1ß, IL-2 and IL-4 are unable to reverse the arrest in the development of $CD4^+$ and $CD8^+$ single positive cells from their double positive precursors (Siegel *et al.*, 1990). Another recent in vitro observation pertinent to the role of the thymus in T cell development is the observation that $CD4^+8^+$ thymocytes bind preferentially to thymic

(medullary) epithelial cell lines and may be eliminated by those cells (Hugo and Potworowski, 1990; Nakashima *et al.*, 1990).

Effects of CyA on the irradiated thymus

The effects of lethal irradiation on the thymus are most prominent at the level of the cortex where most of the thymocytes are lost (Aoyama *et al.*, 1972). Interdigitating cells and macrophages in both cortex and medulla do not disappear (Duijvestijn *et al.*, 1982). On the other hand, induction of clonal deletion (presumably executed by dendritic cells (Lo and Sprent, 1986)) has been shown to be X-irradiation-sensitive whereas induction of clonal anergy is X-irradiation-resistant (Roberts *et al.*, 1990).

Cortical thymocytes are rapidly reconstituted from a syngeneic bone marrow graft (Beschorner *et al.*, 1982). Apparently, the regeneration of the cortex is not significantly influenced by doses of CyA as used in rodent experimental protocols. With respect to CyA-mediated changes in the thymic medulla, irradiation (whether followed by reconstitution or not) does not significantly alter the picture as described for the non-irradiated thymus (Cheney and Sprent, 1985; Beschorner *et al.*, 1987a, 1987c). However, whereas the non-irradiated thymus recovers within about 3 weeks after discontinuation of CyA, the irradiated thymus at 6 weeks after drug withdrawal still lacks medium-sized thymocytes and contains only very few subcapsular thymocytes, medullary histiocytes and medullary thymocytes. Furthermore, MHC class II$^+$ epithelial cells were not detected in the medulla (Beschorner *et al.*, 1987c; Parfrey and Prud'homme, 1990). Thus, although CyA brings about comparable changes in irradiated and non-irradiated thymuses, the main effect of irradiation prior to CyA treatment appears to be on the pace at which the thymus recovers after CyA withdrawal.

T cell maturation in the irradiated, CyA-treated thymus is affected much akin to the way described for the non-irradiated thymus. Hess *et al.* (1987) noticed a relative increase of CD4$^-$8$^-$ double negative cells in the thymus concomitant with a relative decrease of CD4$^+$8$^+$ positive cells as compared with controls. Combined with the virtual absence of single CD4$^+$ cells from the thymus, these authors suggested an incomplete (or shifted) differentiation of T lymphocytes in the thymus of CyA-treated rats. These data were soon thereafter corroborated and extended by studies in mice. Irradiated (8.5 Gy) C57BR mice reconstituted with autologous bone marrow cells were given CyA (20 mg/kg/day) for 23 days. Phenotype analysis of the thymocytes shortly after discontinuation of drug administration revealed inhibition of the development of CD4$^+$ and CD8$^+$ single positive, TCR$\alpha\beta^+$ thymocytes. On the other hand, the development of CD4$^-$8$^-$, TCR$\gamma\delta^+$ thymocytes was not interfered with. Moreover, it was shown that, unlike in controls, cells carrying TCR's of the Vβ17a family [which are potentially (anti-MHC class II) autoreactive in this strain] were not deleted in the CyA treated mice (Jenkins *et al.*, 1988). This study was almost simultaneously confirmed by Gao *et al.* (1988) who showed essentially the same phenom-

enon in 6.0 Gy irradiated (B6 x CBA/Ca)F1 mice which were not reconstituted and given 10 mg CyA/kg/day for 21 days. Also for the mouse strain used in this study, the failure of the thymus to eliminate cells carrying the anti-self MHC class II (I-E) Vß11 TCRs was demonstrated. A protocol comparable to that used in the two last-cited studies was also shown to result in a complete absence of mature T cells from the spleen. Furthermore, in this study no effects were noticed on the generation of $CD4^+8^+$ thymocytes, on the expression of additional surface molecules on $CD4^-8^-$ thymocytes (i.e. CD3, Thy-1, the IL-2 receptor and phagocyte glycoprotein 1 (Pgp-1), and on B cell development (Kosugi *et al.*, 1989a).

The data on the effects of CyA on either the irradiated and the non-irradiated thymus yield a consistent picture. Damage is predominantly inflicted on the medulla and includes both the medullary thymocytes and the stromal cells. The observation that rodents subjected to high-dose irradiation of either the whole body or only the thymus fail to restore the thymic architecture soon after CyA withdrawal suggests a crucial role of the thymic medulla in the development of CyA-AI since non-irradiated animals do not develop disease, although the initial damage to their thymus is comparable (Beschorner *et al.*, 1987c). This suggestion is supported by the finding that CyA-AI is hard to induce in adult rodents (Beschorner *et al.*, 1988d; Fischer and Hess 1990; Bryson *et al.*, 1989). The thymic medulla of adult rodents is less severely affected by CyA and consequently is still present to some extent at the time of discontinuation of CyA (Beschorner *et al.*, 1987c; Parfrey and Prud'homme, 1990).

Damage to the thymus is associated with a virtually complete arrest of differentiation of $CD4^+8^+$ thymocytes into $CD4^+$ and $CD8^+$ single positive cells in the presence of CyA. However, if *in vivo* CyA blood levels decline, especially $CD4^+$ single positive cells reappear in the periphery, and CyA-AI begins to develop (Bos *et al.*, 1988; Fischer *et al.*, 1991). This $CD4^+$ T cell subset is capable to transfer the disease to irradiated secondary recipients (Sorokin *et al.*, 1986). The less striking effect on the $CD8^+$ T cell subset may be due to release of cells intermediary in the development of $CD4^-8^-$ to $CD4^+8^+$, which have the $CD4^-8^+$ phenotype (MacDonald *et al.*, 1988). The work reported by Jenkins *et al.* (1988) and Gao *et al.* (1988) strongly suggests that the medulla of the irradiated thymus may fail to eliminate potentially autoaggressive clones among $CD4^+8^+$ cells when the development of these cells to single positive T cells is resumed after CyA withdrawal. Also experiments in which CD4 molecules were blocked by antibodies or in which transgenic mice were used indicated that deletion of potentially autoaggressive T cells occurs at the $CD4^+8^+$ phenotypic stage (Fowlkes, *et al.*, 1988; Kisielow *et al.*, 1988). Provided normally existent regulatory circuits are eliminated (by irradiation or immunosuppressive chemotherapy (Fischer *et al.*, 1989) or are constitutively absent (like in nude mice; Sakaguchi and Sakaguchi, 1988), CyA-AI may become manifest.

The rapidly growing insight into the normal processes of negative and positive selection of T cells in the thymus opens new perspectives to interpret the disturbance of this process by CyA. The last paragraph will deal with this issue and advance some hypotheses on the mechanisms by which autoreactive cells may cause pathology in CyA-AI as well as on

the induction of the regulatory mechanism normally prohibiting autoreactive cells as active in CyA-AI.

Synthesis and hypotheses

The elegant work of Von Boehmer, Lo, and their colleagues using transgenic mice (Kisielow *et al.*, 1988; Teh *et al.*; 1988; Sha *et al.*, 1988; Van Ewijk, 1990; Von Boehmer, 1990, 1992) has corroborated and extended earlier work on the role of the thymus in tolerance induction ("negative selection") (Kappler *et al.*, 1987, 1988; MacDonald *et al.*, 1988) and the promotion of useful, MHC-restricted, antigen-reactive T cell clones ("positive selection") (Bevan *et al.*, 1977; Zinkernagel *et al.*, 1978; Marusic-Galesic *et al.*, 1989; Zuniga-Pflucker *et al.*, 1989). In both selection processes MHC molecules expressed in the thymus appear to play a crucial role (Kruisbeek *et al.*, 1985; Rouse *et al.*, 1985; Lo and Sprent 1986; Schuurman *et al.*, 1986, Fowlkes, *et al.*, 1988; Salaun *et al.*, 1990). For instance with respect to $CD4^+$ T cells, those showing too strong affinity for self-MHC class II molecules (either alone or in association with a self-peptide) are deleted (Von Boehmer, 1986; MacDonald and Lees, 1990) and those escaping may be extrathymically suppressed or eliminated (Kimura and Wilson, 1984). On the other hand, TCR's able to recognize MHC molecules alone with low affinity (but which might exhibit high affinity for the same molecule in association with an antigenic peptide) are allowed to proceed their development, to leave the thymus and to become part of the T cell repertoire. The specificity of the TCR for thymic MHC class I or class II molecules determines whether the cell will have the $CD4^-8^+$ or $CD4^+8^-$ phenotype, respectively (Von Boehmer, 1986, 1992; Kruisbeek *et al.*, 1988; Sha *et al.*, 1988; Teh *et al.*, 1988).

The marked and persistent reduction of MHC class II antigen expression in the medulla of irradiated young rodents after a course of CyA suggests a causal relationship with the failure of the thymus to delete autoaggressive $CD4^+$ T cells. As long as CyA is administered, the arrest in the development of $CD4^+$ cells might in addition be ascribed to an effect of CyA on TCR-initiated signal transduction for lymphokine synthesis, which may be necessary for T cell differentiation (Gauchat *et al.*, 1986; Bucy *et al.*, 1990; Granelli-Piperno, 1990; Kimball *et al.*, 1990). After withdrawal of CyA the block in T cell development might become weaker while the expression of MHC class II molecules is still defective. Under those circumstances, however, one would expect both positive and negative selection to fail or to proceed incompletely. The failure of the thymus in negative selection is clearly demonstrated in the CyA-AI model. The observation that conventionally bred animals are more likely to die from CyA-AI than specific pathogen-free bred rats (Sorokin *et al.*, 1986) might be interpreted as indicative for a defective positive selection. Another effect of CyA on T cell development was suggested by Shi *et al.* (1989). These authors have demonstrated the inhibition of activation-induced cell death (apoptosis) by CyA. However, if this mechanism would generate the effector cells for CyA-AI, it is

puzzling why the autoaggressive cells do not manifest themselves prior to the disappearance of CyA from the circulation (Bos *et al.*, 1989d). Others have suggested that CyA might keep these cells dormant when already present in the periphery (Wilson, 1989), and we also proposed that autoaggressive T cells are generated during CyA administration, but these inducer/effector cells remain ineffective due to the immunosuppressive activity of CyA (Wodzig *et al.*, 1991; this thesis chapter 5). Further experiments will be needed to dissociate the effects of CyA-mediated reduction of MHC class II antigen expression in the thymic medulla from other effects of CyA on ($CD4^+$) T lymphocyte maturation.

The effects of CyA on the development of $CD8^+$ cells is less clear. Although the development of $CD4^+8^+$ cells to $CD4^-8^+$ cells is blocked under CyA (Jenkins *et al.*, 1988; Gao *et al.*, 1988), this is not as readily reflected in the periphery as the block in $CD4^+$ thymocyte generation (Hess *et al.*, 1987; Bos *et al.*, 1988). This may partly be due to confusion of mature $CD8^+$ cells and immature ($CD3^-$?)$CD8^+$ thymocytes (an intermediate in the transition of $CD4^-8^-$ to $CD4^+8^+$ cells) which may escape from the thymus. Moreover, distinct mechanisms for the induction of tolerance to extrathymic antigens have been suggested for class I-restricted ($CD8^+$) and class II-restricted ($CD4^+$) T cells (Morahan *et al.*, 1989). Therefore, we favour the view that in CyA-AI the primary defect is the failure of the thymus to negatively select precursors of autoreactive $CD4^+$ T cells. We have shown that during CyA therapy autoreactive inducer/effector T cells are generated in and exit from the thymus, because thymectomy immediately after CyA discontinuation could not prevent CyA-AI (Sorokin *et al.*, 1986; Wodzig *et al.*, 1991; this thesis chapter 6). Thus, at the time of CyA withdrawal autoreactive cells reside in a dormant state in the periphery. We suggested that after withdrawal of CyA and the decline of its *in vivo* concentration (Bos *et al.*, 1988) the thymus resumes its function of producing mature $CD4^+$ and $CD8^+$ T cells. Due to the failure of the thymic medulla to timely restore adequate MHC class II expression (Beschorner *et al.*, 1987c), possibly on a certain cell type(s) (Hugo and Potworowski, 1990), $CD4^+$ T cells escape from negative selection. Once these cells enter the periphery they may cooperate with potentially autoreactive lymphocytes which are also present in unmodified individuals. For B cells this assumption is corroborated by data from the model of Sakaguchi and Sakaguchi (1988), in which pathology (by lack of effector T cells?) is predominantly autoantibody-mediated. A comparable mechanism has been proposed to explain humoral immune responses to autoantigens (Mason, 1985). Also in the rat CyA-AI has been demonstrated to be associated with autoantibody responses (Verschuuren *et al.*, 1989; Wodzig *et al.*, 1990; this thesis chapter 4). By analogy, we presume that potentially autoreactive $CD4^+$ (delayed-type hypersensitivity?) and/or $CD8^+$ (cytotoxic) (Hess *et al.*, 1985) T cells (generated during CyA administration) are activated by aberrant $CD4^+$ inducer/helper T cells to become autoaggressive effector cells which respond to target structures expressing self-MHC antigens.

The data from two studies seem to contradict the hypothesis presented above. The mechanism proposed implies that at the time of discontinuation of CyA 1) the thymus should already have generated autoreactive inducer/effector cells of CyA-AI, and 2) that such cells leave the thymus and reside quiescent in the periphery. Data reported by Beschorner *et al.* (1988a) showed that thymocytes from rats with CyA-AI are able to transfer the

disease to secondary recipients. However, these thymocytes were collected 30 days after the discontinuation of CyA in the primary recipients. Therefore, autoreactive ($CD4^+$ helper) T cells are likely to have started their attack on target structures. We explained this discrepancy by the presence of migrating effector cells re-entering the thymus to destroy target cells, i.e. expressing MHC class II antigens (Hess *et al.*, 1985). Furthermore, in the secondary recipients disease became manifest only about 5 weeks after the adoptive transfer. Although at that time the thymuses of the secondary recipients had pathological changes, a considerable period must have been available for generating and exporting T lymphocytes representing the normal TCR repertoire, including potentially autoreactive ones.

The other study which presented data apparently contradicting our hypothesis is that of Cheney and Sprent (1985). These authors showed adoptive transfer of CyA-AI in mice by spleen plus lymph node cells harvested one day after discontinuation of CyA. It was stated that upon adoptive transfer of a non-specified number of lymphoid cells "acute, fatal GVH disease" developed. This observation was presented together with another, quite puzzling one regarding the possibility to transfer CyA-AI with lymphoid cells obtained from non-irradiated mice treated with CyA. However, since primary recipients of CyA did not manifest CyA-AI [probably because the peripheral regulatory circuit(s) were not eliminated (Fischer *et al.*, 1989)] it is hard to understand how transfer of effector cells plus regulatory T cells would permit the development of disease in the secondary recipients. Possibly other factors [e.g. the microbiological status of the mice (Sorokin *et al.*, 1986) influenced the observations made in these transfer experiments. Admittedly, if the data from these experiments were to be reproduced, the model proposed by us would be invalidated.

If one accepts the hypothesis presented above, the search for possible organ-specific target antigens in CyA-AI would become senseless. The finding that pathology (especially in the chronic phase) is confined to certain organs alternatively might be explained by interaction of cell surface molecules involved in homing or recruitment on local endothelial cells and lymphocytes (Berg *et al.*, 1989; Pals *et al.*, 1989). The striking similarity of the pathology in especially the acute phases of CyA-AI and GVH disease would than become rather an example of homology than of analogy. In GVH disease, damage to thymic medullary epithelial cells (Seemayer *et al.*, 1978) may hamper negative selection in a way akin to that was surmised to occur in CyA-AI and consequently yield very similar pathology (Bos *et al.*, 1989c, 1990; Van Bekkum, 1990).

As an alternative to our hypothesis presented above, Sakaguchi *et al.* (1985) have suggested that the pathology in CyA-AI might be caused by $Thy1^+CD4^-8^-$ ($TCR\gamma\delta^+$?) effector cells. Such cells would normally be controlled by autoregulatory $CD4^+$ and $CD8^+$ single positive $TCR\alpha\beta^+$ T cells but might become harmful if these regulatory cells are absent or underrepresented (Sakaguchi and Sakaguchi 1988; Marcos *et al.*, 1988; Heeg *et al.*, 1990). Alternatively, their elimination in the thymus akin to that of $TCR\alpha\beta^+$ autoaggressive T cells might be affected by CyA (Dent *et al.*, 1990). Recently, from a patient with combined immunodeficiency a $CD4^-CD8^-$ $TCR\alpha\beta^+$ T cell clone was isolated; this patient

exhibited GVH-like pathology (Brooks *et al.*, 1990). Both in vivo and in vitro the generation of thymocytes with the $CD4^-8^-$ phenotype has been shown not to be affected by CyA (Jenkins *et al.*, 1988; Kosugi *et al.*, 1989a; Matsuhashi *et al.*, 1989). The export of $CD4^+8^+$ cells from the thymus further suggests the possible release of other, earlier developmental stages including $CD4^-8^-$ thymocytes. Conceivably, their presence in the periphery may be easily overlooked. Therefore, the hypothesis that "uncontrolled" $CD4^-8^-$ $TCR\gamma\delta^+$ and/or $TCR\alpha\beta^+$ cells are causal to the tissue damage in CyA-AI needs further investigation.

Finally, and possibly most importantly in terms of the clinical relevance of the research on these models, the strong autoregulatory circuit(s) which easily nullifies the effects of transfer of CyA-AI effector cells to normal animals should be discussed. Wilson (1989) has drawn attention to the similarity with a autoregulatory circuit revealed in the model of induced GVH resistance using allogeneic cells. In this model, control of GVH reactivity was shown at least in part to be exerted by cytotoxic T cells directed against T cells carrying a TCR reactive with self MHC class II determinants (Kimura and Wilson, 1984a). In this anti-autoreactive circuit, like in that controlling CyA-AI effector cells (Fischer *et al.*, 1989b), both $CD4^+$ and $CD8^+$cells were shown to be involved (Kimura *et al.*, 1984b). However, if such regulatory clones are supposed to be normally elicited in the thymus concomitantly with the generation of positively selected T cells (Sakaguchi and Sakaguchi, 1988), it is difficult to understand why this process would fail to (re)occur in the recovering thymus in CyA-AI. We suggest, in analogy to the model proposed for the activation of autoaggressive effector mechanisms, that the precursors of this suppressive circuit are generated (as part of the repertoire) in the thymus, but that this circuit is only activated in the periphery upon encountering the antigen, e.g. the anti-self MHC TCR. The data of Sakaguchi and Sakaguchi (1988) showing that co-transplantation of normal thymocytes with a *nu/+* thymus from a CyA-treated donor to a *nu/nu* recipient prevents the development of CyA-AI are not necessarily contradictive to this hypothesis. In their model, administration of CyA to the donor thymus is abrupted by its transplantation, so thymic recuperation may proceed much faster than observed after withdrawal of CyA in the same host. Consequently, suppressor circuit precursors may be generated in time to prevent CyA-AI. Thus, the data presently available do not contradict "anti-idiotypic" (autoregulatory) responses to autoreactive T cells in the peripheral lymphoid organs of unmodified animals. In CyA-AI, where these organs are initially atrophic, the expansion and the effects of autoaggressive clones may have proceeded too far before this counteractive circuit can become adequately activated.

The discussion above confirms that the model of CyA-AI is very intriguing and will in the near future definitely contribute to our understanding of T cell maturation and selection in the thymus. Moreover, it may help to elucidate the mechanisms (in control) of GVH disease and autoimmunity.

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Chapter 2

Susceptibility and resistance to Cyclosporine A - induced autoimmunity in Rats

Susceptibility and resistance to Cyclosporine A - induced autoimmunity in rats

Abstract

Lethally irradiated Lewis (LEW) rats, reconstituted with syngeneic bone marrow and next given Cyclosporine A (CyA) for several weeks, develop disease (Cyclosporine A-induced autoimmunity; CyA-AI) after withdrawal of CyA. This disease resembles in terms of dermal changes the acute dermatitis and chronic scleroderma also seen in graft-versus-host disease (GVHD). In this study we report the relative resistance of the Brown Norway (BN) rat strain to the induction of CyA-AI.In contrast to LEW rats, in which CyA-AI was originally described, BN rats showed no acute dermatitis or scleroderma-like skin pathology in spite of comparable changes in the thymus and a maturation arrest of $CD4^+$ T cells. The difference was also demonstrated functionally for whereas in LEW rats delayed-type hypersensitivity (DTH) reactions could not be elicited during CyA-AI, these were within normal limits in BN rats subjected to the same protocol; NK activity on the other hand was unaffected in both strains. The observation that BN rats developed very mild late disease as evidenced by a slight though significant weight loss suggests that the BN strain is relatively susceptible to the disease but that lesser effector cell generation or, alternatively, stronger suppressor cell responses may prevent dermal disease. These observations may contribute to the elucidation of the mechanisms involved in this experimental autoimmune disease.

Introduction

Cyclosporine A (CyA) is a drug with potent immunosuppressive activity and effective in preventing allograft rejection (White and Calne, 1982; Shevach, 1985; Hess *et al.*, 1986), suppressing graft-versus-host disease (GVHD) secondary to allogeneic bone marrow transplantation (BMT) (Tutschka *et al.*, 1979; Bacigalupo *et al.*, 1990), and in the treatment of several autoimmune diseases (Stiller *et al.*, 1984; Bolton *et al.*, 1982; Von Graffenried, 1989). On the other hand, CyA administration after lethal X-irradiation and reconstitution with syngeneic or autologous bone marrow may, 2-3 weeks after withdrawal of CyA, elicit a T cell-mediated autoimmune syndrome with pathology similar to that seen in acute GVHD after allogeneic BMT (Glazier *et al.*, 1983). This CyA-induced autoimmune syndrome was originally termed syngeneic GVHD (Glazier *et al.*, 1983) and has also been referred to as CyA-induced autoimmune disease (CyA-AI) (Sorokin *et al.*, 1986) or BMT-associated immune disease (BMT-ID) (Bos *et al.*, 1990. The disease has been elicited in certain rat (Glazier *et al.*, 1983) and mouse strains (Cheney and Sprent, 1985; Bryson *et al.*, 1989) and may also occur in humans (Jones *et al.*, 1989). The rat model as originally described proved to be easily reproducible using young LEW ($RT1^l$) rats; males and females are equally susceptible (Glazier *et al.*, 1983; Fischer and Hess, 1990). In two other studies, female Louvain (Lou/M; $RT1^a$) and (LEWxBN)F1 rats were shown to be susceptible as well (Tutschka *et al.*, 1987; Geller *et al.*, 1989); we also reproduced the model using female DA ($RT1^a$) rats. This paper confirms the susceptibility of LEW rats to CyA-AI and documents the resistance of BN ($RT1^n$) rats to this acquired autoimmune disease.

Materials and methods

Animals

Female, specific pathogen-free LEW ($RT1^l$) and BN ($RT1^n$) rats were used at the age of 4-6 weeks. Rats were obtained from our own breeding stock.

Protocol for induction of CyA-AI

The experimental protocol has been described before (Bos *et al.*, 1988). In brief, rats were given a lethal dose of 8.5 Gy at 0.5 Gy/min using a Röntgen irradiation machine (Philips MG320, Hamburg, Germany) and next day received a syngeneic BMT (see below). Starting from the day of BMT rats received 7.5 mg CyA/kg/day for 42 days. CyA (kindly donated by Sandoz Co. Ltd., Basel, Switzerland) was dissolved in olive oil at a concentration of 7.5 mg/ml and administered subcutaneously. CyA-AI usually developed 2-3 weeks after cessation of CyA administration. LEW or BN rats subjected to this protocol are referred to as LEW/XCyA and BN/XCyA.

Bone marrow transplantation

Bone marrow donor rats were killed by cervical dislocation under ether anesthesia. Bone marrow was collected from tibias and femurs in Dulbeccos balanced salt solution supplemented with 2% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) (DBSS). Recipient rats received $6x10^7$ viable syngeneic bone marrow cells in 1.0 ml DBSS intravenously into a tail vein.

Scoring of macroscopic pathology

After withdrawal of CyA, development of CyA-AI was assessed by examination of the rats 1-2 times a week for signs and symptoms of disease. Macroscopic pathology usually developed in the following order: red acra (hyperemia of the skin of the ears, feet, and tail), inflammation of the eyelids, dermatitis, and alopecia (Table 2.1). Furthermore, rats were weighed daily to assess changes in body weight. Loss of body weight in LEW rats with CyA-AI corresponds with the severity of dermal signs of disease and dermal histopathological changes (Wodzig *et al.*, 1991). Change in body weight was expressed as percent of body weight determined on the day of BMT. Skin biopsies were taken on day 0, 21, 56, and 84 after withdrawal of CyA.

***Table 2.1:** Coding of macroscopic pathology in CyA-AI.*

Code	Pathology
E	Erythroderma of the acra (hyperemia of the skin of the ears, feet and tail)
D	Dermatitis, usually starting around the eyes and with brown/red discoloring of the fur of the neck, front and/or hind legs. Expansion is initially restricted to the ventral part of the body.
A_v	Ventral alopecia
A_g	Generalized alopecia
T	Thin fur, usually dorsal (consequence of slowly progressive disease or recovery from alopecia)

Control groups

Three different control groups were included in this study: X-irradiated and bone marrow-transplanted LEW and BN rats given olive oil (LEW/XOil and BN/XOil); age-matched LEW and BN rats treated with CyA (cyaLEW and cyaBN); age-matched non-treated LEW and BN rats (nLEW and nBN).

Immunoperoxidase

Thymuses were frozen in cold isopentane and 4 µm sections were cut, air dried, and fixed in aceton for 10 min. Slides were air dried and incubated with an optimal dilution of mouse monoclonal antibodies (MAbs) in PBS/0.05% BSA for 60 min at room temperature. The specific MAb from ascites fluid or supernatant consisted of OX6 (RT1.B, MHC class II antigen), OX17 (RT1.B, MHC class II antigen), OX18 (RT1.A, MHC class I antigen), OX19 (CD5, panspecific for rat T lymphocytes), OX62 (rat dendritic cells), R73 (T cell receptor αβ), and ED-1 (rat macrophages, dendritic cells). Antibodies in the OX series were kindly donated by Dr. A. Williams, Oxford, UK except for OX62 (kindly provided by Dr. M. Brenan, Oxford, UK); ED-1 (by Dr. C. Dijkstra, Amsterdam, The Netherlands); and R73 (by Dr. T. Hünig, Heidelberg, Germany). The slides were washed 3 times with cold PBS followed by incubation with horse radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG, to which 3% normal rat serum was added. After washing the slides 3 times with cold PBS, a substrate containing 0.05% diaminobenzidine (DAB) and 0.05% H_2O_2 in 50 mM Tris-HCl pH 7.6 was added. The slides were counterstained with hematoxylin, dehydrated, and coverslipped with Entallan (Merck, Darmstadt, Germany).

Langerhans cell identification

Langerhans cells in the skin were identified by ATP-ase staining and immunoperoxidase staining for rat MHC class II antigens (Thorbecke *et al.*, 1980). Skin biopsies were frozen in cold isopentane and 4 µm sections were cut, air dried, and fixed either in neutral formalin-$CaCl_2$-Macrodex for ATP-ase staining or in aceton for 10 min for immunoperoxidase staining.

ATP-ase: Slides were washed with aqua bidest and incubated for 45 min at 42°C with 0.05% adenosin-5-triphosphate/0.08 M Tris(HCl)-malein acid buffer pH 7.2/0.12% $Pb(NO_3)_2$/0.02 M $MgSO_4$. Control slides were incubated with the same solution without adenosin-5-triphosphate. The slides were washed with water, incubated 30 sec with 2% Na_2S, and washed with water. Thereafter, slides were stained for 10 min with eosin, rinsed with water, dehydrated with aceton and diethylether, and mounted with Entallan.

Immunoperoxidase: Slides were washed 3 times with cold PBS and incubated with an optimal dilution of MAb OX6 and treated according to the protocol of immunoperoxidase staining (see above).

Cytofluorometry

Peripheral blood lymphocytes from BN rats were analysed or the relative concentrations of $CD4^+$ and $CD8^+$ T cells, and B cells on day 35 after BMT (under CyA), and on days 60 and 82 days after BMT (18 and 40 days after discontinuation of CyA). Cells were labeled using mouse MAbs to rat CD4 (W3/25), CD8 (OX-8), IgM (MARK, kindly donated by Dr. G. Voisin), and goat $F(ab')_2$ anti-mouse IgG-FITC absorbed with rat Ig (Cappel/Organon Teknika, Turnhout, Belgium) as described before (Bos *et al.*, 1988). Cytofluorometry was performed on a Becton-Dickinson FACScan flow cytometer using LYSYS II software.

Cell-mediated immune reactivity

Cell-mediated (type IV) hypersensitivity to a contact allergen was elicited according to Wijsbek et al. (1991). In brief, rats were sensitized to toluene di-isocyanate (TDI; Merck Schuchardt, Hohenbrunn, Germany) by two epicutaneous applications. For the first sensitization a total volume of 500 µl 5% TDI solution was stepwise applied along the whole length of the tail. One week later 500 µl 5% TDI solution was applied to a shaven area on the back of the rat. Control rats received the solvent only. Seven days after the second immunization, experimental rats were challenged under anesthesia by application of 20µl 1% TDI solution to the outside of the left ear. Just prior to challenge measurements of left ear thickness were performed using a digital micrometer (Digimatic type ID-110M; Mitutoya, Tokyo, Japan). Each determination was performed 10 times; standard deviations of series of 10 determinations did not exceed 10% of the mean. Increment of ear thickness was expressed as percent of ear thickness determined prior to challenge. Control rats were challenged in the same fashion, but in fact encountered the allergen for the first time. The respons to TDI was expressed as the mean increase of ear thickness (MIET) of experimental rats (n=5) minus the MIET of control animals (n=3).

Natural killer (NK) cell activity

Blood samples of approximately 1.0 ml were obtained by retro-orbital puncture and added to 50 IE heparin (Leo Pharmaceuticals, Weesp, The Netherlands). Peripheral blood leukocytes (PBL) were recovered by lysis of the erythrocytes with 25 ml 0.84% NH_4Cl and centrifugation (30xg; 10 min) of the suspension on a cushion of 5 ml iFCS. Pelleted cells were washed and the suspension adjusted to contain $5x10^6$ viable PBL/ml.

Five million K562 (target) cells were suspended in 0.2 ml RPMI 1640 with 2% iFCS and 7.4 MBq $Na_2{}^{51}CrO_4$ and incubated at 37°C for 60 min. After labelling the target cells were washed and adjusted to a concentration of $5x10^4$ cells/ml in RPMI 1640 with 10% iFCS. A range of 100 to 3-fold excess of PBL was added to $5x10^3$ ^{51}Cr-labeled target cells in a final volume of 0.2 ml medium per round-bottom microtiter well. All determinations were set up in duplicate. Microtiter plates were centrifuged (30xg, 5 min) and incubated for 6.5 hrs at 37°C in air with 5% CO_2. Thereafter, the plates were centrifuged again and the supernatants collected by absorption (Skatron Harvesting System, Costar, Cambridge, USA). Radioactivity present in the supernatants was measured in a gamma counter. Maximum release of ^{51}Cr was determined by incubation of $5x10^3$ target cells with 100µl Cetavlon shampoo. Percent specific lysis was calculated according to the formula:

$$\text{\% specific release} = \frac{\text{cpm experimental release - cpm spontaneous release}}{\text{cpm maximum release - cpm spontaneous release}} \times 100$$

Statistics

Differences in the weights of rats from different groups were tested for statistical significance by Wilcoxons rank sum test. Statistical analysis of cell-mediated hypersensitivity responses was also performed by Wilcoxons rank sum test.

Results

Development of CyA-AI in LEW versus BN rats

Female LEW (n=30) and BN (n=27) rats were treated for induction of CyA-AI (LEW/XCyA and BN/XCyA); control LEW (n=20) and BN (n=19) rats received X-irradiation, sBMT and the CyA-solvent only (LEW/XOil and BN/XOil). On day 20 after withdrawal of CyA the mean body weight of the LEW/XCyA rats had decreased 14% when compared to the mean body weight on day 0 (range of individual weight losses: 5-30%). In contrast, the mean body weight of LEW/XOil rats showed an increase of 4% as compared to day 0 (range of individual weight gains: 1-9%). The difference in mean body weight of LEW/XCyA and LEW/XOil rats was statistically significant on day 14 after CyA withdrawal and remained so for the duration of the experiment ($P<0.001$)(Fig. 2.1).

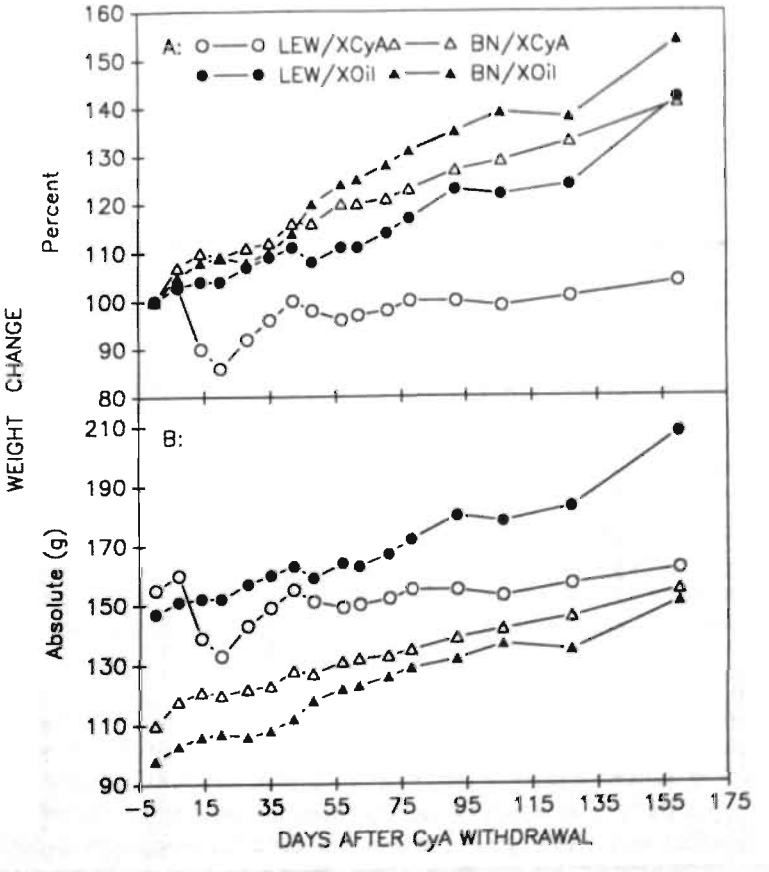

Figure 2.1.: Weight changes of LEW/XCyA, BN/XCyA, and control LEW/XOil and BN/XOil rats after CyA withdrawal. Panel A: Mean relative weights as compared to the weight on the day of CyA discontinuation (day 0 = 100%); panel B: Mean absolute weights in g.

On day 20 after cessation of CyA administration the group of BN/XCyA rats showed a mean increase in body weight of 9% as compared to day 0 (range 2-15%). BN/XOil rats showed a comparable weight gain over the same period (mean: 9%; range 5-17%). From day 40 after CyA withdrawal a slight but significant difference in the relative mean body weights of BN/XCyA and BN/XOil rats developed ($P<0.04$)(Fig. 2.1).

On day 20 after CyA withdrawal 26 out of 29 experimental LEW/XCyA rats exhibited severe macroscopic skin pathology like dermatitis (6/26) and alopecia (20/26); one rat showed symptoms associated with the onset of CyA-AI like erythroderma of the acra whereas two rats showed no macroscopic signs of CyA-AI (Table 2.2). From day 20 after cessation of CyA all LEW/XCyA rats eventually developed skin pathology; in most of the animals dermatitis and ventral alopecia progressively evolved to generalized alopecia. LEW/XOil rats showed no signs of disease over the same period. ATP-ase staining and anti-MHC class II immunoperoxidase staining of skin biopsies from LEW/XCyA and LEW/XOil rats showed comparable densities of stained cells in biopsies from both groups.

BN/XCyA and BN/XOil rats for the period of observation of over 160 days showed no signs of CyA-AI, although in some of the BN/XCyA rats the fur was more grayish than that of BN/XOil animals. Microscopic examination of skin biopsies obtained from BN/XCyA rats revealed no abnormalities when compared to biopsies taken from BN/XOil animals.

Table 2.2: *Development of CyA - induced macroscopic skin pathology in LEW/XCyA rats.*

Day[a]	N[b]	Macroscopic pathology of CyA-AI[c]					
		absent	E	D	A_v	A_g	T
0	30	30					
6	30	27		3			
14	30	8		11	11		
20	29	2	1	6	20		
28	29	3			24	2	
35	29	4			10	11	4
42	27	2			12	9	4
48	26	2			3	15	6
57	26	2			1	18	5
62	26	2			2	18	4
71	25	2			2	17	4
78	25	2				18	5
92	25	1				19	5
106	25	1				19	5
160	24					21	3
169	24					21	3
181	24					21	3
189	23					20	3
202	23					20	3

[a]After discontinuation of CyA. [b]Loss of animals occurred accidentally due to manipulation. [c]E, erythroderma; D, dermatitis; A_v, ventral alopecia; A_g, generalized alopecia; T, thin fur (see also Table 2.1).

Effect of CyA treatment on thymic architecture

Thymic samples obtained on days 14 and 28 after BMT and on day 56 after BMT (14 days after withdrawal of CyA) from LEW/XCyA, LEW/XOil, and cyaLEW were compared with BN/XCyA, BN/XOil, and cyaBN by histology and immunohistochemistry. As compared to LEW/XOil and BN/XOil, thymuses from LEW/XCyA and cyaLEW as well as BN/XCyA and cyaBN showed a marked ablation of the medulla during CyA administration (days 14 and 28 after BMT), which was associated with loss of epithelium resulting in markedly reduced MHC class II antigen expression. Furthermore, lymphocytes with a mature T cell phenotype (high TCR$\alpha\beta^+$) and dendritic cells were also reduced due to medullary involution. Thymic cortical areas appeared hardly affected by CyA treatment. On day 56 after BMT (14 days after cessation of CyA) changes in thymuses of cyaLEW, LEW/XCyA, cyaBN, and BN/XCyA rats had reversed to normal, but persisted in thymuses of LEW/XCyA and BN/XCyA rats.

Phenotypic analysis of BN peripheral blood lymphocytes

Lymphocytes from BN/XCyA (n=6), BN/XOil (n=6), cyaBN (n=4), and nBN (n=4) rats were subjected to FACS analysis on day 35 after BMT (under CyA administration) and on days 60 and 82 after BMT (18 and 40 days after discontinuation of CyA) (Fig.2.2). On day 35 relative numbers of both $CD4^+$ and $CD8^+$ cells were suppressed in BN/XCyA, cyaBN, and BN/XOil rats as compared to nBN rats, albeit most strikingly so for $CD4^+$ cells in BN/XCyA (4.9% versus 45%). At day 82 after BMT, relative numbers of $CD4^+$ and $CD8^+$ cells had recovered to near-normal values. Consequently, $CD4^+$ to $CD8^+$ ratios remained nearly constant (3.5 to 4.6) for all groups except for BN/XCyA rats in which the ratios were 1.1 (day 35), 1.8 (day 60) and 3.6 (day 82) respectively.

Cell-mediated immunity in LEW versus BN rats

Although the absence of pathology from BN/XCyA rats treated according to the standard protocol suggested relative resistance to CyA-AI in this strain, the decrease in weight observed subsequently suggested a low grade disease to be present. In order to verify whether BN skin differed from LEW also in terms of function, DTH reactions were elicited during CyA-AI. LEW/XCyA, LEW/XOil, BN/XCyA, and BN/XOil rats randomly selected from these groups were tested at 6, and other rats at 15 weeks after cessation of CyA (or solvent) administration. Responses were compared to those of age-matched nLEW and nBN rats.

Five nLEW and 5 LEW/XOil rats taken at 6 weeks post-CyA showed comparable DTH reactivity. At 24 hrs after challenge, thickness of the challenged ears had strongly increased (MIET: nLEW rats 65%; LEW/XOil rats 81%) and declined over the next 48 hrs. Five LEW/XCyA rats taken randomly at 6 weeks post-CyA (and all suffering of CyA-AI) mounted no response over 72 hrs after challenge (Fig. 2.3A). Five LEW/XCyA rats taken randomly at 15 weeks, however, showed a weak response that peaked at 48 hrs (MIET: 27%). This response at 48 hrs was not significantly different from the response at that time point in LEW/XOil and nLEW rats (Fig. 2.3B).

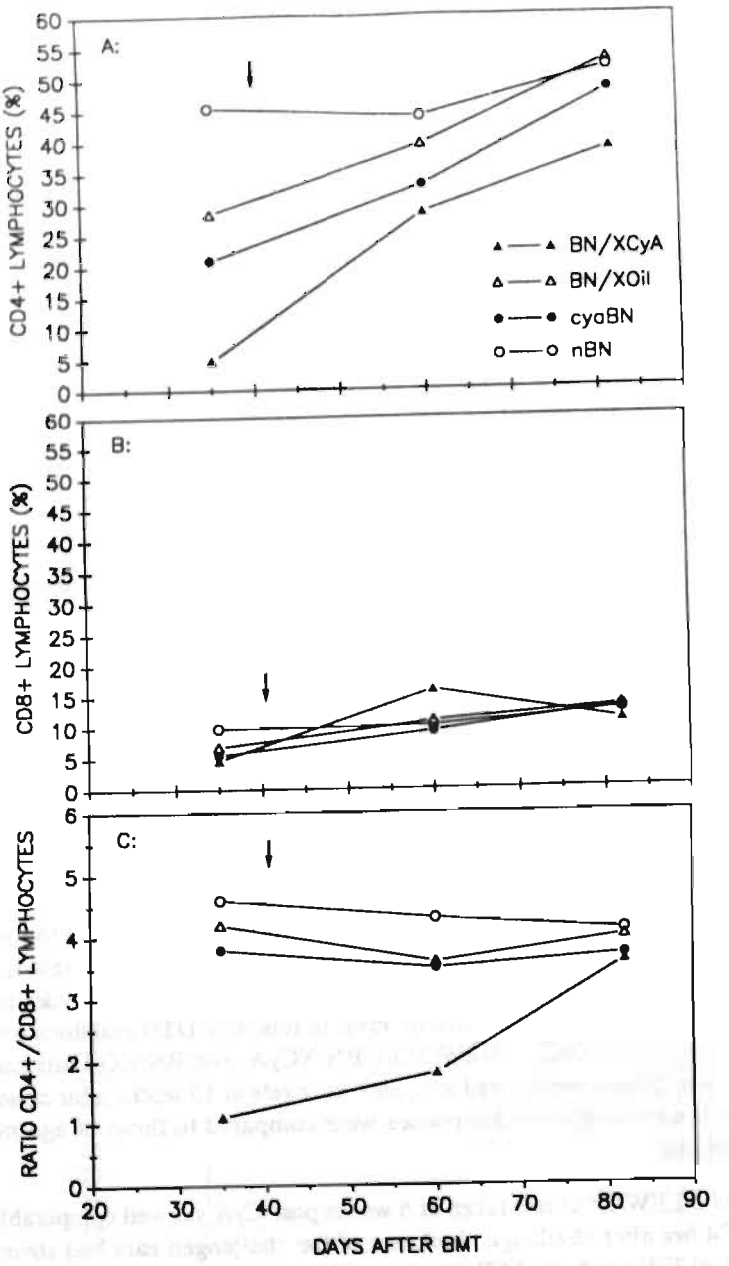

Figure 2.2: Repopulation of lymphocyte subsets in peripheral blood during and after cessation of CyA or solvent administration. ▲, *BN/XCyA: X-irradiated, BMT, BN rats given CyA for 42 days;* △, *BN/XOil: X-irradiated, BMT, BN rats given olive oil for 42 days;* ●, *cyaBN: age-matched BN rats treated with CyA for 42 days;* ○, *nBN: age-matched non-treated BN rats. Panel A: percentage of CD4⁺ lymphocytes (T-helper); panel B: percentage of CD8⁺ lymphocytes (T-cytotoxic/suppressor); panel C: ratios of CD4⁺ to CD8⁺ lymphocytes. Arrows indicate day of CyA withdrawal.*

DTH responses of nBN rats were significantly lower than that of nLEW rats ($P<0.03$). Furthermore, responsiveness in nBN rats appeared to decline with age (MIET in 18 weeks old rats: 44%, and in 27 weeks old rats: 25% at 24 hrs). Five BN/XOil rats taken at 6 weeks after discontinuation of the solvent (aged 18 weeks) displayed a significantly weaker response than 5 nBN rats (MIET 20% at 24 hrs; $P<0.04$), but this difference was not found at 15 weeks. In two groups of 5 BN/XCyA rats taken at 6 and 15 weeks after cessation of CyA strongest DTH responses were detected at 24 hrs after challenge (MIET 9% and 23%, respectively). These responses were not significantly different from those of BN/XOil rats. A statistically significant difference was, however, detected between responses at 48 hrs after challenge of BN/XCyA and BN/XOil rats taken at 15 weeks after CyA administration ($P<0.04$)(Fig. 2.3B).

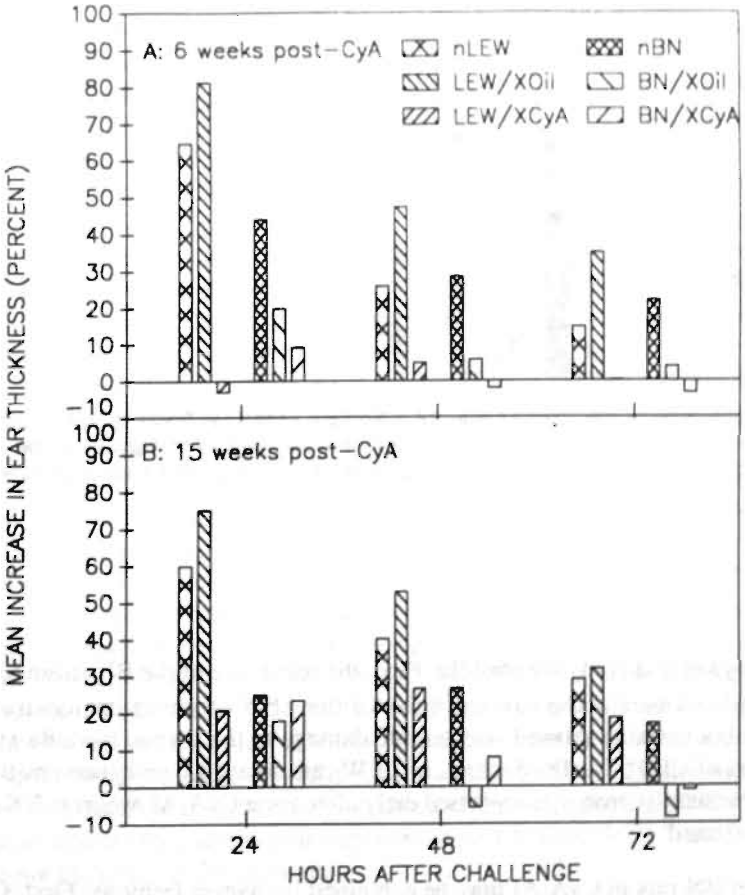

Figure 2.3: DTH to TDI in the left ears of LEW/XCyA, BN/XCyA, LEW/XOil, BN/XOil, nLEW and nBN rats (for explanation see legend to Fig. 2.2). DTH was monitored by ear swelling at 24, 48 and 72 hrs after challenge. Panel A: DTH to TDI at 6 weeks after CyA discontinuation; panel B: 15 weeks after discontinuation.

NK-activity

Since weak cell-mediated immunity in some models appears to be compensated for by NK cell activity, this parameter was investigated as well (Herberman, 1982). NK-activity was determined in PBL 8 weeks after CyA withdrawal. At an effector:target cell ratio of 100:1 NK-activity in 5 LEW/XCyA rats (mean specific lysis 8.1%; rang 4-13.3%) was not significantly different of that of 2 LEW/XOil (mean specific lysis 6.4%; values 5 and 7.7%) and 2 age-matched nLEW rats (mean specific lysis 5.4%; values 5.0 and 5.7%)(Fig. 2.4).

In 5 BN/XCyA rats NK activity (mean specific lysis 5.8%; range 1.4-13.1%) was not significantly different of that in 3 BN/XOil rats (mean specific lysis 4.3%; range 2.2-7.9%) and 2 age-matched nBN rats (mean specific lysis 8.1%; values 6.2 and 9.9%) (Fig. 2.4).

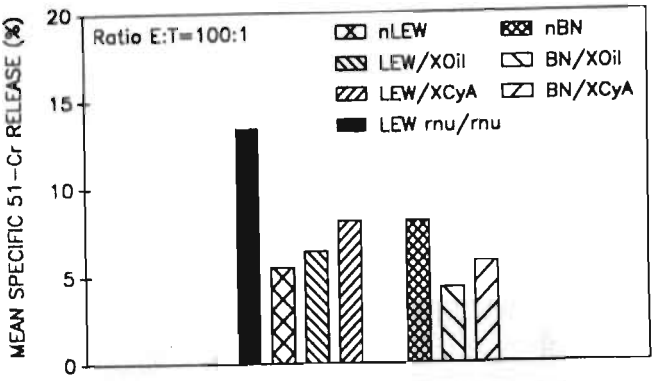

Figure 2.4: NK cell activity in PBL from LEW/XCyA, BN/XCyA, LEW/XOil, BN/XOil, nLEW and nBN rats at 8 weeks after CyA discontinuation (for explanation see legend to Fig.2.2). In the same experiment, for comparison NK cell activity in PBL from a LEW r nu/r nu congenic rat was determined.

Discussion

The main findings of this study are twofold. First, the resistance of the BN strain to the induction of CyA-AI *versus* the susceptibility of the LEW strain is demonstrated, although rats of both strains showed comparable damage to the thymic medulla and a maturation arrest of $CD4^+$ T cells. Second, in LEW rats delayed-type hypersensitivity reactions were transiently strongly suppressed early after acute CyA-AI, whereas NK cell activity was unaffected.

The resistance of BN rats to CyA-AI may be explained in diverse fashions. First, CyA metabolism in BN and LEW rats may differ. This is an unlikely explanation, however, since CyA is equally effective in suppressing first set renal allograft rejection in both LEW and BN recipients (Steinbrüchel *et al.*, 1991; Brüning *et al.*, 1989). Furthermore, in both

LEW and BN rats CyA caused a comparable ablation of the medulla during administration, which was associated with loss of epithelium, dendritic cells, lymphocytes with a mature T cell phenotype (high TCR$\alpha\beta^+$), and MHC class II antigen expression. These thymic changes are reversed after cessation of CyA. Thymic cortical areas appeared not to be affected by CyA treatment. Comparable thymic changes are described for CyA-treated LEW (Beschorner *et al.*, 1987a, 1991), PVG ($RT1^c$) and WAG/CPB ($RT1^u$) rats (Schuurman *et al.*, 1990). Furthermore, in BN rats CyA caused a maturation arrest of T cells as evidenced by a marked reduction of peripheral $CD4^+$ T cells during CyA administration and a rebound of these cells only after cessation of CyA administration. In the LEW model of CyA-AI, a comparable delayed recovery of the $CD4^+$ T lymphocyte subset under CyA administration has been described (Bos *et al.*, 1988; Fischer *et al.*, 1991). In LEW rats this rebound of $CD4^+$ cells is associated with the onset of disease, regardless of the duration of CyA administration (Bos *et al.*, 1988, 1989; Fischer *et al.*, 1991).

Recently it has been shown that in the rat the $CD4^+$ T cells can be divided into two subpopulations based on the expression of the antigens CD45RC (as recognized by monoclonal antibody OX-22) and RT6. The $CD45RC^+RT6^-$ subset of rat $CD4^+$ T cells has a T helper 1 (T_H1)-like lymphokine repertoire (high IL-2 and IFNγ production but low IL-4) and provides cell-mediated immune responses, whereas the $CD45RC^-RT6^+$ population has characteristics of T helper 2 (T_H2)-type cells (the provision of B cell help, IL-4 production, and suppression of cell-mediated responses but low IL-2 and IFNγ production) (Fowell *et al.*, 1991; Fowell and Mason, 1993). Furthermore, it has been demonstrated in a model for diabetes that the $CD45RC^+RT6^-$ subset contains the autoagressive cells, whereas the $CD45RC^-RT6^+$ subset inhibits this autoimmune potential (Fowell and Mason, 1993). In this context, it is interestingly to see that normal BN rats (known to respond in a T_H2-like fashion) have a preponderance of the T_H2-like subset over T_H1, whereas for LEW rats (known to respond in a T_H1-likefashion) the opposite is the case (Groen *et al.*, 1993). The repertoire of cytokines produced can promote the development of either T_H1- or T_H2-type responses. Most significantly, IL-4, a product of T_H2-type T cells, inhibits the induction of T_H1-type responses, suggesting that a potent T_H2 reaction, as present in BN rats, is likely to decrease cell-mediated immunity (Fowell and Mason, 1993). By analogy, adoptive transfer of unfractionated $CD4^+$ T cells from untreated BN rats to syngeneic secondary irradiated recipients would not lead to induction of autoimmune disease because this unfractionated population in normal BN rats consists of a mixture of predominating $CD45RC^-RT6^+$ cells (T_H2-like) with autoregulatory (suppressor) activity and inferior $CD45RC^+RT6^-$ cells (T_H1-like) with autoreactive potential in a ratio of 28:1, whereas in LEW rats the $CD4^+$ T cells showed a preponderance of $CD45RC^+RT6^-$ (autoreactive) cells (Groen *et al.*, 1993). We assume therefore that in the BN strain which was resistant to induction of CyA-AI, the $CD45RC^-RT6^+$ T cells were able to regulate the pathological responses of the $CD45RC^+RT6^-$ T cell population, which was not the case for the LEW rats.

Second, BN rats may lack the target antigen(s) to which the autoimmune effector cells are directed. MHC class II molecules possibly represent one of these antigens, since in

rats with acute CyA-AI cytotoxic T cells directed to public MHC class II epitope(s), including self-epitopes, have been demonstrated (Hess *et al.*, 1985). This explanation, however, is also unlikely since apart from LEW also Lou/M rats (Tutschka *et al.*, 1987), (LEWxBN)F1 rats (Geller *et al.*, 1989), and DA rats (personal communication) are susceptible to induction of CyA-AI. There is no reason to assume that MHC class II antigen expression in BN rats would be significantly different from that in the susceptible rat strains. Susceptibility of LEW and (LEWxBN)F1 rats, taken together with the resistance of BN rats, suggests that susceptibility is genetically a dominant trait.

Third, resistance of BN rats to induction of CyA-AI might be due to weaker cell-mediated effector mechanisms leading to skin disease as compared to LEW. Others have shown a preponderance of CD4 over CD8 cells in cutaneous lesions in CyA-AI (Beschorner *et al.*, 1988), which suggests that DTH effector T cells may be involved in bringing about the skin lesions.

Our data demonstrate that the dermal pathology of CyA-AI in LEW evolves even in the absence of DTH reactivity. On the other hand normal numbers of Langerhans cells were observed. Others have shown that CyA abolishes MHC class II expression on dendritic cells in the thymus, but does not influence peripheral MHC class II expression (Schuurman *et al.*, 1990). DTH non-responsiveness in LEW rats could be due to dermal pathology or, alternatively, to absence of $CD4^+$ DTH effector T cells. The latter hypothesis is unlikely since peripheral $CD4^+$ T cells increased upon withdrawal of CyA and $CD4^+$ T cells, presumably involved in mediating dermal changes, can be found at the site of the lesions in the skin (Bos *et al.*, 1988; Fischer *et al.*, 1991; Beschorner *et al.*, 1988). In accordance with the former hypothesis, also in man, skin DTH reactions may be decreased in scleroderma patients, even when the antigen is applied subcutaneously (Glinsky and Jablonska, 1985). In contrast, others failed to demonstrate generalized defects of immune responsiveness in patients with systemic sclerosis (Lupoli *et al.*, 1990). Finally, NK activity in LEW and BN rats was similar and not influenced by CyA-AI. Therefore, NK cell activity is unlikely to be involved in the mechanism(s) explaining resistance of BN rats to induction of CyA-AI.

The fourth and last hypothesis to be forwarded here rests on our observation that administration of CyA for either 3, 6, or 12 weeks does not increase the severity of CyA-AI after drug withdrawal (Bos *et al.*, 1988). Apparently, when the potentially autoreactive T cells generated under CyA administration have left the thymus during the first 2-3 weeks after BMT, even under prolonged CyA administration abberant negative selection in the thymus is corrected, although intrathymic MHC class II molecule expression remains suppressed (Beschorner *et al.*, 1987b, 1988a, 1988b). This might be effected by the intrathymic generation of a T cell population counteracting the autoreactive T cells (Kimura and Wilson, 1984; Wilson, 1989). If this intrathymic balance would earlier be restored in the BN strain than in CyA-AI susceptible strains, the net effect in the BN strain would be a failure to release enough autoreactive effector T cells to cause CyA-AI.

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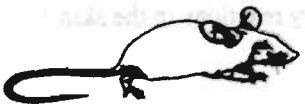
Chapter 3

Malignant neoplasms in Cyclosporine A-induced autoimmunity



Malignant neoplasms in Cyclosporine A - induced autoimmunity

Abstract

Lethally irradiated LEW rats reconstituted with syngeneic bone marrow and given Cyclosporine A (CyA) for 6 weeks following syngeneic bone marrow transplantation (BMT) develop, 2-3 weeks after withdrawal of CyA, symptoms of disease resembling graft-versus-host disease (GVHD) as seen after allogeneic bone-marrow transplantation. Symptoms of disease include acute dermatitis, chronic disease resembling scleroderma, and occasionally antibody-mediated myasthenia gravis.

This study shows that animals treated according to this protocol may, after cessation of CyA administration, in addition develop malignant neoplasms. In one experiment, 27 out of 35 experimental animals developed rapidly progressive subcutaneous and/or intracutaneous tumors commencing at 6 weeks (n=1), 13 weeks (n=1), and about 6 months (n=25) after cessation of CyA. None of the 21 control animals developed tumors during a follow-up period of over one year. In 19 experimental rats mesenchymal tumors, mostly high grade sarcomas, were present, and in 5 adenocarcinomas. The majority of tumors expressed either class I or both class I and class II MHC antigens. Tumors were both as explants and as cell suspensions transferable to syngeneic LEW but not to allogeneic BN, and congenic LEW.1N or BN.1L rats, demonstrating that both major and minor histocompatibility antigens are required for tumor growth. This study shows that malignant neoplasms may arise as a late complication of CyA-induced, thymus-dependent autoimmune disease. The genesis of these malignancies might be explained by the conjunction of X-irradiation and thymus (T cell)-dependent immune-mediated chronic inflammatory reactions in the skin.

Introduction

Cyclosporine A (CyA) has been shown to be an effective immunosuppressive agent acting primarily on T lymphocyte-dependent immune responses. It has been used extensively to suppress allograft rejection (Shevach, 1985; Hess *et al.*, 1986), acute graft-versus-host disease (GVHD) associated with allogeneic bone marrow transplantation (BMT) (Tutschka *et al.*, 1979; Bacigalupo *et al.*, 1990) and treatment of several autoimmune diseases, i.e. diabetes mellitus, multiple sclerosis and lupus erythematosus (Stiller *et al.*, 1984; Von Graffenried *et al.*, 1989). By contrast, administration of CyA after X-irradiation and syngeneic bone marrow transplantation (BMT) may induce thymus-dependent autoimmune disease (CyA-AI) resembling GVHD as seen after allogeneic BMT (Glazier *et al.*, 1983). For example, lethally X-irradiated LEW rats reconstituted with syngeneic bone marrow and given CyA for 5 weeks following BMT develop, usually 2-3 weeks after withdrawal of CyA, symptoms of disease. These symptoms may include acute disease resembling GVHD, chronic disease resembling scleroderma, and antibody-mediated disease like myasthenia gravis. This phenomenon (also referred to as syngeneic GVHD) can be reproducibly elicited in young animals of certain rat and mouse strains (Fischer *et al.*, 1990; Cheney and Sprent, 1985; Bryson *et al.*, 1989), and may also occur in humans (Jones *et al.*, 1989). The central role of the thymus in CyA-AI has been unequivocally established. Shielding of the thymus during total-body X-irradiation (TBI), as well as thymectomy prior to TBI, prevented the induction of CyA-AI (Sorokin *et al.*, 1986).
The unmodified thymus plays a key role in the generation of T lymphocytes involved in immune surveillance and cytotoxic activity *versus* foreign and/or transformed cells (Melief and Kast, 1991). Under the experimental conditions described, CyA has been reported to disturb induction of tolerance to self. In our model, CyA is only administered transiently during the period of recapitulation of thymic ontogeny after X-irradiation, resulting in an arrest of maturation of thymic T cells to CD4 and CD8 single positive cells (Beschorner *et al.*, 1987; Gao *et al.*, 1988; Jenkins *et al.*, 1988; Hiramine *et al.*, 1989; Kosugi *et al.*, 1989; Fischer *et al.*, 1991) and in aberrant negative selection (Hess *et al.*, 1985; Hess and Fischer, 1989). The development of CyA-AI appears to be associated with the reappearance of $CD4^+$ T cells in the peripheral blood about two weeks after withdrawal of CyA (Bos *et al.*, 1988; Fischer *et al.*, 1991) and/or the induction of cytolytic T lymphocytes (CTLs) recognizing a public MHC class II determinant (Hess *et al.*, 1985). On the basis of this observation the model of CyA-AI has been propagated in man as a therapeutic manoeuvre for treating class II MHC positive hematopoietic neoplasms, e.g. non-Hodgkin's lymphomas (Jones *et al.*, 1989; Hess *et al.*, 1992).

We now report, paradoxically, the development of malignant neoplasms as a late symptom of CyA-AI. In the present study we have investigated some parameters like the incidence, onset, and type of tumors, and we addressed the question whether development of tumors was related to X-irradiation, CyA administration, or the presence of CyA-AI.

Materials and methods

Animals

Female, specific pathogen-free (SPF) Lewis (LEW, $RT1^l$) and BN ($RT1^n$) rats were used. LEW rats were purchased from Charles River (Sulzfeld, Germany) and BN rats were obtained from our own breeding stock (Department of Central Animal Facilities, University of Limburg, Maastricht). Congenic female LEW.1N (possessing the BN MHC on the LEW genetic background) and BN.1L rats (genetically reciprocal to LEW.1N) were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. Animals were used at the age of 4-6 weeks.

Protocol for induction of CyA-AI

LEW rats were given 8.5 Gy at 0.5 Gy/min. using a Röntgen irradiation machine (Philips MG320, Hamburg, Germany) one day prior to syngeneic BMT. Bone marrow(BM) donor rats were killed by cervical dislocation under ether anaesthesia. BM was collected from tibias and femurs in Dulbeccos balanced salt solution supplemented with 2% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) (DBSS). Recipient rats received $6x10^7$ viable syngeneic bone marrow cells in 1.0 ml DBSS intravenously into a tail vein. CyA, a gift from Sandoz Co. Ltd., Basel, Switzerland, was dissolved in olive oil at a concentration of 7.5 mg/ml. Rats were weighed daily and received 7.5 mg/kg/day CyA subcutaneously for 6 weeks starting on the day of BMT. CyA-AI usually developed 2-3 weeks after cessation of CyA administration (Wodzig *et al.*, 1991).

Design of the experiment

A cohort of 35 experimental LEW rats received X-irradiation, syngeneic BMT, and CyA and are referred to as LEW/XCyA (Table 3.1). Age-matched control LEW rats (n=21) included 7 rats that received X-irradiation, syngeneic BMT, and olive oil (LEW/XOil); 7 rats that received only CyA (cyaLEW); and 7 non-treated rats (nLEW).

Scoring of macroscopic pathology

After withdrawal of CyA, development of CyA-AI was assessed by examination of the rats twice a week for signs and symptoms of disease: red acra (hyperemia of the skin of the ears, feet and tail), inflammation of the eyelids, dermatitis, alopecia and weight loss (Wodzig *et al.*, 1991).

Histology of tumors

Tumors were isolated from rats treated for induction of CyA-AI by sterile surgical procedures. Tumor fragments were either fixed in 4% neutral buffered formalin, embedded

in paraffin, and stained with hematoxylin and eosin or frozen in isopentane at -70°C for immunohistochemical studies.

Immunohistochemistry

Frozen tumor fragments were cut at 4 µm, fixed in cold acetone, and air dried. Sections were washed 3 times for 5 min with PBS. Incubations were performed at room temperature in wet chambers. Sections were incubated for 60 min with primary mouse monoclonal antibodies (MAbs) diluted in phosphate buffered saline (PBS) with 0.05% bovine serum albumin (BSA) according to previously determined magnitude. Primary MAbs included OX18 (detecting rat MHC class I antigen, RT1.A), OX6 (rat MHC class II antigen, RT1.B), RECA-1 (rat endothelial cells), MDE II (human desmin; cross-reactive with rat desmin and localizes desmin in tumors derived from muscle tissue), and MVI (bovine vimentin; cross-reactive with rat and staining vimentin in fibroblasts, endothelial cells, lymphoid tissue and melanocytes as well as tumors derived from such cells). Furthermore, a rabbit antiserum (Z311) directed to bovine S-100 A and B antigens (cross-reactive with rat and human S-100 A and B antigens present in glial and ependymal cells in the brain, Schwanns cells of the peripheral nervous system, skin melanocytes, and Langerhans cells) was used. Antibodies of the OX series were kindly donated by Dr. A. Williams, Oxford, UK and RECA-1 by Dr. A.M. Duijvestijn, Maastricht, The Netherlands. MAbs to desmin and vimentin were purchased from Organon Teknika (Oss, The Netherlands) and the antiserum Z311 from DAKO (Glostrup, Denmark). After incubation with primary antibodies, sections were washed with PBS and incubated with horse radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) diluted in PBS supplemented with 3% normal rat serum. After washing with PBS, sections were stained by incubation with 0.05% diaminobenzidine (DAB) and 0.06% hydrogen peroxide in 50 mM Tris-HCl pH 7.6 for 10 min. Sections were counterstained with hematoxylin, dehydrated, and mounted with Entallan.

Transfer of sarcoma explants and cell suspensions

LEW sarcomas were cut in cubicles of 3x3x3 mm and 2 fragments were transplanted subcutaneously to the left and 2 to the right side of syngeneic LEW, LEW.1N, BN.1L and BN rats. In case of tumor "take", increased tumor size was usually palpable 2-3 weeks after transplantation. Animals were monitored for tumor growth up to 2 months after transplantation. In case no tumor growth was detected by palpation, obduction was performed two months after transplantation to confirm failure of tumor "take". For single cell suspensions, LEW sarcomas were cut in cubicles of 5x5x5 mm and incubated with collagenase type I (200 U/mg; Sigma, St.Louis, USA) and DNase I (400-600 U/mg; Sigma) in 10 ml Eagles medium for 20 min at 37°C under gentle rotation. Thereafter, 35 U/ml hyaluronidase type IV (800-2,000 U/mg; Sigma) was added for 5 min to complete dissociation. The digest was poured through nylon gauze screens (100 µm mesh) to separate single cells from non-dissociated tumor fragments. The resulting single cell suspension was centrifuged for 10 min, 490 g, at 4°C, washed 2x with Hanks's Balanced Salt Solution (HBSS) and cell viability assessed by trypan blue exclusion. Five to 60 million tumor cells were transferred to LEW rats by subcutaneous injection at each flank.

Statistics

Differences in the weights of rats from different groups were tested for statistical significance by Wilcoxons rank sum test.

Results

Induction of CyA-induced autoimmunity (CyA-AI)

A group of 35 experimental LEW rats was treated according to the protocol for induction of CyA-AI (LEW/XCyA); control LEW rats were included in the experiment (LEW/XOil, cyaLEW, and nLEW). After discontinuation of CyA animals were monitored for signs and symptoms of disease as described above. On day 7 after cessation of CyA, both the LEW/XCyA and all control LEW rats on average had gained 2% and 3% in body weight respectively as compared to day 0. On day 14 after withdrawal of CyA, the mean body weight of the LEW/XCyA rats had dropped 1% as compared to the mean body weight on day 0 (range of individual weight change: -15% to +4%). In contrast, the mean body weight of all control rats showed an increase of 5% as compared to day 0 (range of individual weight gain: 1-10%)(Fig. 3.1). The difference in mean body weight of LEW/XCyA and pooled controls was statistically significant on day 14 after CyA withdrawal and remained so for the duration of the experiment ($P<0.001$). The weight loss of the LEW/XCyA rats in the acute phase of disease coincided with the appearance of the clinical symptoms of CyA-AI.

Twenty-three out of 35 (66%) LEW/XCyA rats developed macroscopic symptoms of CyA-AI 2-4 weeks after withdrawal of CyA. Seven weeks after cessation of CyA, 32 out of 35 (91%) LEW/XCyA rats showed signs of CyA-AI. None of the controls developed any macroscopic abnormality (Table 3.1).

Incidence of malignant neoplasms in CyA - AI

During follow-up of the LEW/XCyA animals for 12 months 27 out of 35 rats (77%) developed neoplasms, whereas in 21 controls no tumors were found. Within the LEW/XCyA group, tumors occurred with comparable incidence in rats showing no symptoms of CyA-AI (2/3; 66%), in rats with severe alopecia (20/24; 83%), and in rats with partial alopecia (thin fur)(5/8; 63%). The earliest development of neoplasms, however, was observed in rats progressing to full alopecia after an episode of acute dermatitis, at 6 (n=1) and 13 weeks (n=1) after cessation of CyA respectively; all other tumors were observed more than 24 weeks after cessation of CyA (Table 3.2).

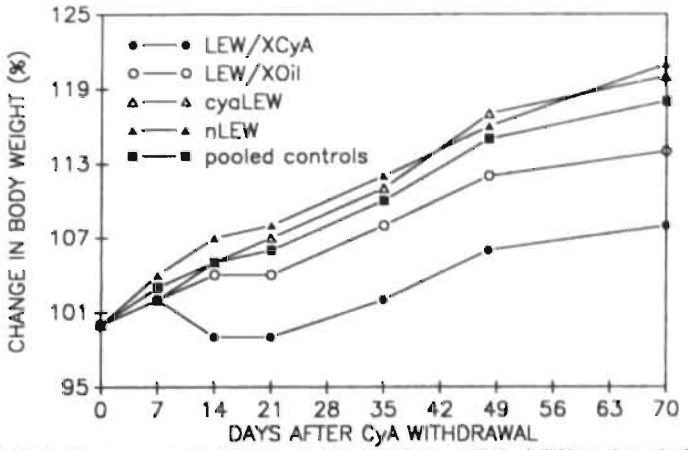


Figure 3.1: Weight changes of LEW/XCyA, LEW/XOil, cyaLEW, nLEW, and pooled controls after CyA withdrawal. *Mean relative weights are shown as compared to the weight on the day of CyA discontinuation (day 0 = 100%). From day 14 onward LEW/XCyA versus pooled controls $p<0.001$ and for LEW/XCyA versus LEW/XOil $p<0.05$.*

Table 3.1: *Design of the experiment and incidence of CyA - induced autoimmune disease.*

Group	N	Treatment[a]		Onset of CyA-AI[b]		
		X-irr + sBMT	CyA	acute[c]	subacute[c]	absent
Experimental						
LEW/XCyA	35	+	+	23	9	3
Controls						
LEW/XOil	7	+	-			7
cyaLEW	7	-	+			7
nLEW	7	-	-			7

[a]X-irr, X-irradiation; sBMT, syngeneic bone marrow transplantion; CyA, Cyclosporine A administration (7.5 mg/kg/day for 5 weeks). [b]CyA-AI was established by erythroderma, dermatitis, alopecia, and weight loss. [c]Acute: within 4 weeks after cessation of CyA; subacute: more than 4 weeks after withdrawal of CyA.

Table 3.2: *Incidence and onset of neoplasms in CyA - AI.*

Group	N	Tumor	Onset of tumor after CyA withdrawal		
		Incidence (%)	6 weeks	13 weeks	>24 weeks
Controls	21	0/21 (0)			
Exp. LEW/XCyA					
No symptoms of CyA-AI	3	2/3 (66)			2[a]
Alopecia	24	20/24 (83)	1	1	18
Thin Fur	8	5/8 (63)			5
All LEW/XCyA	35	27/35 (77)			

[a]One of these tumors is an (radiation-induced)osteosarcoma; due to extensive necrosis of the second tumor no histological diagnosis could be made.

Characterization of Malignant Neoplasms in CyA-AI

Histopathology

Subcutaneous and/or intracutaneous tumors developed in 27 LEW/XCyA rats. Mesenchymal tumors were present in 19 rats, epithelial tumors in 5 rats, and an epithelial as well as a mesenchymal tumor in 2 rats. Due to extensive necrosis, in one case no distinct histological diagnosis of the tumor could be made.

Of the 19 rats developing a mesenchymal tumor, one was a benign neurofibroma. The others were malignant with characteristics of, usually high grade, sarcomas showing histological patterns of malignant fibrous histiocytoma (MFH)(n=5)(fig. 3.2), fibrosarcoma (n=4)(fig. 3.3), round cell sarcoma not otherwise specified (n=2), malignant schwannoma (n=2; characterized by histology and abundant expression of the S-100 antigen), osteosarcoma (n=1), and rhabdomyosarcoma (n=4; characterized by histology and expression of desmin) (Table 3.3). The osteosarcoma and the tumor without diagnosis had developed in rats with no clinical symptoms of CyA-AI.

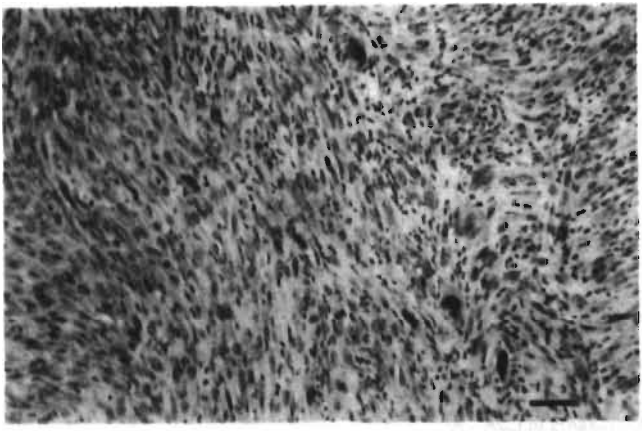


Fig 3.2: Malignant fibrous histiocytoma; giant cells in a spindle cell tumor (Bar represents 50 μm)

Fig 3.3: Fibrosarcoma; spindle cell tumor (Bar represents 50 μm)

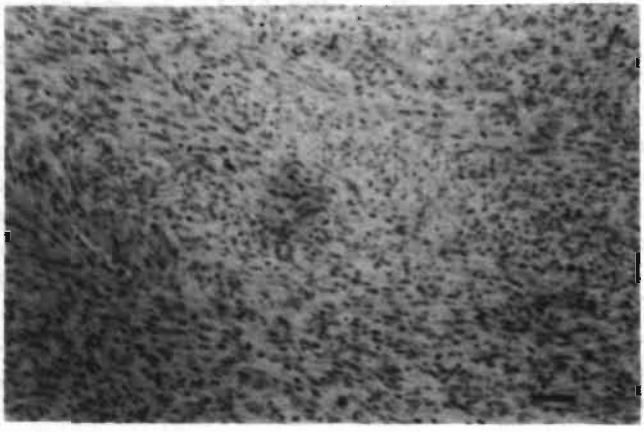


Table 3.3: *Characterization of tumors in LEW/XCyA rats.*

Tumor type (total tumors in each (subigroup)	N
Mesenchymal (19)	
benign neurofibroma	1
high grade sarcoma (18)	
malignant fibrous histiocytoma (MFH)	5
fibrosarcoma	4
round cell sarcoma not otherwise specified	2
malignant schwannoma	2
osteosarcoma[a]	1
rhabdomyosarcoma	4
Epithelial (5)	
sebaceous carcinoma	2
syringocystadenocarcinoma	1
adenoid cystic carcinoma	1
carcinoma not further classified	1
Mesenchymal + Epithelial (2)	
benign fibroadenoma + fibrosarcoma	1
sebaceous carcinoma + round cell sarcoma	1
No diagnosis (1)[a]	1

[a] *The osteosarcoma and the tumor without diagnosis developed in LEW/XCyA rats with no symptoms of CyA-AI (see Table 3.2).*

The epithelial tumors were all adenocarcinomas (n=5) presumably of skin appendageal origin. These tumors showed features of sebaceous carcinoma (n=2), syringocystadenocarcinoma papilliferum (n=1), adenoid cystic carcinoma in (n=1), whereas the remaining adenocarcinoma could not be further classified. Of the 2 rats that developed a mesenchymal as well as a separate epithelial tumor, one had a benign epithelial tumor localized in the breast, showing features of a fibroadenoma, and a fibrosarcoma. The other rat developed a tumor with characteristics of a sebaceous carcinoma and a separate mesenchymal tumor specified as a round cell sarcoma.

All tumors developed as rapidly progressive subcutaneous and/or intracutaneous tumors at different sites (Table 3.4). Tumor localization was not related to the sites of CyA injection. Tumors did not metastasize to other organs as verified macroscopically at autopsy, with the exception of the osteosarcoma which was prominent in the knee-joint and had metastasized to the kidney and stomach.

Immunohistochemistry

Mesenchymal tumors were heterogeneous with respect to MHC antigen expression. Eleven out of 18 high grade sarcomas expressed only MHC class I (60%), 5 expressed both MHC class I and class II (28%), 1 expressed only MHC class II (6%), and 1 - the osteosarcoma - expressed neither MHC class I nor class II antigens (6%). MHC antigens

Table 3.4: Localization and characterization of neoplasms in CyA - AI.

Localization	N	Mesenchymal tumor[a]							Epithelial	Mes + Ep
		NF	MFH	FS	RCS	SW	O	Rh	Tumor	Tumor[b]
Medial/lateral										
right side[c]	11		3			1		4		2
left side	4		2		2					
Head	1			1						
Hind leg/anus	3								3	
Hind leg	2			1			1			
Front leg	4	1		1		1			1	
Chest/neck	2			1					1	
Total tumors	27									

[a]*NF, neurofibroma; MFH, malignant fibrous histiocytoma; FS, fibrosarcoma; RCS, round cell sarcoma; SW, schwannoma; O, osteosarcoma; Rh, rhabdomyosarcoma.* [b]*Mes + Ep, both mesenchymal and epithelial tumor.* [c]*The tumor without diagnosis developed at the right side.*

could not be detected on benign neurofibroma cells. Four out of 5 epithelial tumors expressed only MHC class I antigen (80%), and 1 expressed both MHC class I and II antigens (20%)(Table 3.5).

Table 3.5: MHC antigen expression of neoplasms in CyA - AI.

Tumor type	N	Incidence of MHC expression in tumors			
		class I	class II	class I/II	none
Mesenchymal					
Neurofibroma	1				1 (100)[a]
Sarcoma	18	11 (60)	1 (6)	5 (28)	1 (6)
Epithelial					
Adenocarcinoma	5	4 (80)		1 (20)	

[a]*() percentage of tumors expressing either MHC or no MHC antigens.*

Mesenchymal and epithelial tumors were well vascularized as assessed by staining with the MAb RECA-1.

All epithelial tumors showed cytoplasmic expression of keratin, whereas all mesenchymal tumors had cytoplasmic expression of vimentin. In addition, malignant schwannomas expressed the S-100 antigen, the rhabdomyosarcomas expressed desmin.

Transfer of LEW sarcoma to different rat strains

LEW sarcomas were transplanted to syngeneic LEW, allogeneic BN, and congenic LEW.1N and BN.1L rats. Growth of the explant was usually palpable 2-3 weeks after transplantation in syngeneic LEW, but not in allogeneic BN, congenic LEW.1N and BN.1L rats. BN, LEW.1N and BN.1L rats were checked for tumor growth up to 2 months after transplantation and obduction at two months confirmed failure of tumor "take" (Table 3.6).

Sixty million LEW fibrosarcoma cells were transferred as cell suspension to LEW, BN, LEW.1N, BN.1L, and immunocomprized (X-irradiated, BM-reconstituted) LEW and BN rats. LEW fibrosarcomas were transferable both as explants and as cell suspensions to untreated and immunocomprized LEW rats, but not to LEW.1N, BN.1L, BN or immunocomprized BN rats (Table 3.6).

***Table 3.6** : Passive transfer of LEW sarcoma to different rat strains.*

Strain	LEW/X[a]	LEW	LEW.1N	BN.1L	BN/X[a]	BN
MHC	I	I	n	I	n	n
Genetic background	I	I	I	n	n	n
Tumor "take"	+	+	-	-	-	-

[a]LEW/X and BN/X rats are lethally irradiated and reconstituted with syngeneic bone marrow cells.

Discussion

The main findings of this study are fourfold. First, a substantial number of LEW rats treated for induction of CyA-AI (LEW/XCyA) developed malignancies, notably sarcomas including malignant fibrous histiocytoma (MFH). The latter is a tumor of heterogenous histogenesis with a widely dispersed immunohistochemical profile; the etiology is still unknown (Dehner, 1988). Second, these soft-tissue tumors developed late in the natural history of CyA-AI and seem to be associated with cutaneous disease. Third, the majority of the tumors expressed to a variable extent either MHC class I or both MHC class I and class II antigens. Finally, the osteosarcoma excepted, no metastases were observed.

Although the pathogenesis of the neoplasms observed in this study is obscure, possible factors included the way in which the rats were X-irradiated, the dose of syngeneic bone marrow administered, the dose and duration of CyA therapy, and the strain of rats used. There is circumstantial evidence to support the hypothesis that in the experimental setting used by us, these neoplasms are specific for CyA-AI for two reasons. First, no

malignancies were observed in X-irradiated and syngeneic bone marrow-reconstituted animals nor in CyA-treated rats; this suggests (1) an indirect role for the manipulated thymus in the pathogenesis of these tumors and/or (2) interference of CyA with DNA-repair after X-irradiation. Second, to our best knowledge there are no other rodent models which describe these tumors after X-irradiation and/or CyA administration. Although Hess *et al.* (1989, 1992) also used the model of CyA-AI, they used different dosages of X-irradiation and CyA; this resulted usually in progressive disease within two to four weeks after cessation of CyA and thus - compared to our model - less chronicity of lesions (Beschorner *et al.*, 1988b).

Although in man tumors may develop following therapeutic or accidental X-irradiation, these are usually epithelial tumors, lymphoreticular neoplasms, and bone tumors (Hajdu, 1979; Land, 1987; Smith, 1987). Sarcomas have a tendency to occur in areas previously exposed to ionizing irradiation but arise uncommon in the field of X-irradiation; of the sarcomas osteosarcomas are most commonly induced by X-irradiation (Smith, 1987; Chang *et al.*, 1989). In other mammalian species the types of neoplasms observed after X-irradiation may be different from those listed above for man. The types of neoplasms induced may be X-irradiation dose-dependent (Upton, 1984) but tumors as described here for the model of CyA-AI have not been observed after X-irradiation alone.

A potent immunosuppressant like CyA also may have considerable side-effects; in general immunosuppression is often associated with increased incidence of malignancies (Penn, 1987). In man, the incidence of lymphoproliferative disorders and other malignant diseases is higher in CyA-immunosuppressed patients than in the population as a whole (Ryffel, 1992). The effect of CyA on carcinogenesis has been addressed in animal studies following the observation in man that high dosages of CyA given to renal allograft patients induced (non-Hodgkin's) lymphomas (Calne *et al.*, 1979). In OF-1 mice and OFA rats high dosages of CyA (between 8 and 16 mg/kg) administered for 78 weeks or longer as a single agent did not increase tumor incidence when compared to controls (Ryffel *et al.*, 1983). However, when mice were manipulated by X- or ultraviolet light (UV)-irradiation prior to chronic administration of CyA, a reduction in the latency period of lymphoma and/or cutaneous squamous cell carcinoma was observed, but the incidence of tumors was not increased (Hattori *et al.*, 1987, 1988; Kelly *et al.*, 1987). In contrast, another study failed to demonstrate a synergistic effect of X-irradiation and CyA administration (up to 100 mg/kg) on either latency or incidence of murine lymphoma (Hooghe *et al.*, 1989). In rats, chronic administration of CyA accelerated the growth of chemically-induced gastrointestinal tumors (Ryffel, 1992). In man, in the setting of allogeneic kidney transplantation, CyA treatment may increase the incidence of non-Hodgkin's lymphoma, squamous cell carcinoma of the skin, and Kaposi's sarcoma in this order (Penn, 1987; Penn and Brunson, 1989), but in the therapeutic doses used nowadays the incidence of these tumors is similar to that seen with other immunosuppressive regimes like azothioprine and prednisone (Ryffel 1992). Thus, possibly by virtue of its immunosuppressive effect chronic CyA administration may enhance tumor growth in some experimental models of disease and may elicit tumor growth in human allograft recipients (Penn, 1987; Penn and Brunson, 1989; Ryffel 1992).

How then might the tumors in our model of CyA-AI be generated, and why did the tumors not metastasize? The model of CyA-AI has been advocated as an anti-tumor treatment for a variety of hematologic (lymphohematopoietic) malignancies and solid tumors in man (Hess *et al.*, 1992) because in this model cytotoxic lymphocytes can be demonstrated *in vitro* directed against public MHC class II determinants (Jones *et al.*, 1989). Also in Lou/M rats it has been shown that $CD8^+$ T cells from animals with symptoms of acute CyA-AI exerted *in vitro* tumor reactivity against a syngeneic plasmacytoma line which expressed MHC class II antigens (Hess *et al.*, 185; Geller *et al.*, 1989). The model of CyA-AI was also assessed for anti-tumor effects in C57bl/6 mice. Mice were subjected to X-irradiation, syngeneic bone marrow transplantation, and transient CyA therapy; although these mice did not develop clinically manifest CyA-AI they were capable of rejecting MHC class II bearing tumors provided interleukin-2 (IL-2) was infused as well (Charak *et al.*, 1991). In contrast to the rat model, however, the IL-2-dependent anti-tumor effect was not mediated by cytotoxic T cells directed against MHC class II antigens, but by $Thy1^+$, asialo GM^+ cells. Furthermore, the antitumor effect of these killer cells was non-specific and not restricted to the tumor of the host only, unlike that seen with CyA alone. Thus, in this model two mechanisms that exert anti-tumor effects appear to be operative: IL-2 therapy supports the generation of killer cells with non-MHC-restricted cytotoxicity against most tumor cells, but not normal tissue (Charak *et al.*, 1991), whereas CyA-AI induces killer cells with specificity against public MHC class II determinants on both host and tumor cells (Hess *et al.*, 1985; Jones *et al.*, 1989; Geller *et al.*, 1989; Hess *et al.*, 1992).

Since the malignant tumors observed in our study expressed class I and/or class II MHC antigens (with the exception of the osteosarcoma with metastases) and because increased NK activity is present in the model of CyA-AI when compared to normal LEW rats both in the absence- (Wodzig *et al.*, 1993) and presence of tumors, we suggest that the absence of metastases in MHC positive sarcomas is due to high NK activity and possibly anti-MHC class II cellular cytotoxicity (Herberman, 1983; Hess *et al.*, 1985, 1992; Reiter *et al.*, 1991). NK-activity in a LEW/XCyA rat with a fibrosarcoma was increased 4-6 times (specific lysis 33.8%) as compared to LEW/XCyA and nLEW rats (mean specific lysis 8.1% and 5.4% respectively)(data not shown).

How the tumors were generated is obscure but we offer the following explanation. Our hypothesis is based on the observation that no tumors were elicited in X-irradiated or CyA-treated rats, but that chronic inflammatory skin disease was present in 93% (25 out of 27) of all animals with tumors, the rat with the osteosarcoma excepted. Malignant neoplasms often develop in areas of chronic scarring, ulceration, or sinus formation, such as burn scar, chronic osteomyelitic sinus, decubitus ulcers, and in skin areas affected by lupus erythematosus (DeVita et al., 1989). With respect to inflammatory reaction and collagen deposition chronic CyA-AI shows strong similarity to human scleroderma, a frequently occurring manifestation of progressive systemic sclerosis (PSS)(Bos *et al.*, 1989). In the acute phase of CyA-AI pathology encompassed diffuse infiltrates of mononuclear cells in the dermis, especially round small hair follicles in the upper dermis, and at the dermal/epidermal junction. Furthermore, myositis was frequently observed.

Later on, folliculitis was the most conspicuous abnormality and affected small and large hair follicles in the dermis and subdermal fat. Fibrosis of the subdermal region usually developed around hair follicles with an inflammatory reaction. As the disease progressed from acute to chronic, the diffuse inflammation of the dermis decreased, but inflammation of hair follicles persisted; most prominently in hair follicles situated in the lower dermis or the subdermal fat. In the upper dermis, the number of hair follicles was reduced. In the dermis hyperplasia of sebaceous glands was frequently observed and fibrosis still progressed. The majority of rats with chronic CyA-AI showed persistence of the inflammatory reaction, manifesting as a folliculitis in the subdermal fat, and as an increase of collagen in the subcutaneous region. A minority of rats with chronic CyA-AI showed no longer inflammatory reactions, but an increase of collagen and complete absence of subcutaneous fat only; some rats showed no signs of chronic disease (Bos *et al.*, 1989). The presence of these scleroderma-like lesions in chronic CyA-AI rats may have contributed to and enhanced growth of these neoplasms; malignant fibrous histiocytoma showed similarities to the "fibrohistiocytoid"cells in chronic inflammation (Imai *et al.*, 1989) and the genesis of the adenocarcinomas may also be related to inflammatory skin-reactions around hair follicles and sebaceous glands. Furthermore, several observations support a relationship between cancer (carcinoma) and PSS, like occurrence of breast carcinoma at or near the time of onset of scleroderma (Lee *et al.*, 1983), increased incidence of lung cancer (bronchogenic carcinoma) (Roumm *et al.*, 1985), and rarely alveolar cell carcinoma (Talbot *et al.*, 1979).

In the experimental model of CyA-AI tumors could be transplanted as explants or cell suspensions to normal LEW rats where they showed progressive, non-metastatic growth. This observation argues against the hypothesis that interference with thymic programming (resulting in aberrant immunosurveillance) (Melief and Kast, 1991) in conjunction with X-irradiation-mediated DNA damage initiating a process of transformation causing the growth of sarcomas and to a lesser extent epithelial tumors. This view, in addition, is refuted by the fact that these tumors grow well in normal LEW rats with an unmanipulated thymus.

In conclusion, our hypothesis holds that in this experimental model tumors were caused by a combination of X-irradiation and a chronic - cutaneous - inflammatory reaction and in this fashion were indirectly thymus-dependent. Further experiments are required to elucidate the mechanisms operative in this model.

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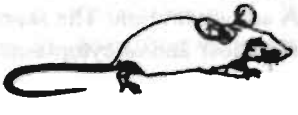
Chapter 4

Autoantibodies in Cyclosporine A-induced experimental autoimmune scleroderma



Autoantibodies in Cyclosporine A - induced experimental autoimmune scleroderma

Abstract

Lethally irradiated Lewis (LEW) rats given syngeneic bone marrow and Cyclosporine A (CyA) for 6 weeks develop, after withdrawal of CyA, acute and chronic graft-versus-host diseaselike symptoms (CyA-induced autoimmunity, CyA-AI). Dermal lesions in rats with chronic CyA-AI are clinically and histologically similar to those of limited scleroderma in man. In patients with rheumatic diseases, circulating autoantibodies against nuclear or cytoplasmic antigens are frequently demonstrable. For example, 30% of patients with diffuse systemic sclerosis have anti-DNA-topoisomerase I (Scl-86) antibodies, and 70 to 80% of the patients with limited scleroderma develop anticentromere/kinetochore (CENP- or CR19) antibodies. The present study was performed to examine by means of Western Blots whether the sera of rats with CyA-AI and scleroderma-like lesions contained autoantibodies to Scl-86 and/or CR19; and if so, whether these are associated with the scleroderma-like lesions observed in CyA-AI. In this study we report that human autoantibodies to Scl-86, 56K, SS-B/La, and B'/B antigens, but not to CR19, crossreacted with rat nuclear and cytoplasmic antigens of the same molecular weights. Furthermore, multiple autoantibodies to human nuclear and cytoplasmic antigens of various M_w's ranging from 20 to 100 kD were only demonstrable in sera from experimental LEW rats with manifest CyA-AI disease but not prior or during CyA administration. The rat cell fractions recognized by these rat autoantibodies are nuclear and/or cytoplasmic proteins of various M_w's

ranging from 37 to 100 kD. None of the sera from LEW rats with chronic CyA-AI, obtained on different weeks after withdrawal of CyA, showed autoantibodies comparable to those with diagnostic importance in human-mediated "connective tissue" diseases, i.e. Scl-86 or CR19. Thus, in this model the lesions of scleroderma appear to be dissociated from the presence of antibodies to Scl-86 or CR19. There was a striking resemblance with respect to the presence of autoantibodies between LEW rats with CyA-AI (to which the BN strain is resistant) and BN rats with mercurychloride ($HgCl_2$)-induced autoimmune nephritis (to which LEW are resistant). However, $HgCl_2$-treated BN rats developed hyperimmunoglobulinemia and showed elevated relative concentrations of γ-, β-, and α1-globulins as compared to LEW rats with CyA-AI. Apparently, in the rat different autoimmune models may elicit antibodies to similar nuclear and cytoplasmic proteins, suggesting that analogous autoimmune mechanisms are involved.

Introduction

Scleroderma or (progressive) systemic sclerosis (SS) is a connective tissue disease characterized by fibrosis and degenerative changes in the skin, small arteries, several internal organs, and immunologic abnormalities (Bernstein *et al.*, 1982). SS is believed to have an (auto)immune origin, although no clear pathogenetic relationships have been defined. The reasoning for an (auto)immune pathogenesis was based on immunogenetic associations, similarities between SS and chronic graft-versus-host disease, and cell-mediated and humoral immune abnormalities in SS patients like decreased numbers of circulating T lymphocytes, hypergammaglobulinemia and the occurrence of auto-antibodies (Livingston *et al.*, 1987).

Lesions analogous to those seen in scleroderma have been observed in association with a graft-versus-host reaction after allogeneic bone marrow transplantation in man (Shulman *et al.*, 1978), but also after autologous bone marrow transplantation if the patient received ablative chemotherapy and/or total body irradiation prior to transplantation (Jones *et al.*, 1989; Hess *et al.*, 1992).

In the experimental rat model of Cyclosporine A (CyA)-induced syngeneic graft-versus-host disease (sGVHD)(Glazier *et al.*, 1983), also referred to as CyA-induced autoimmunity (CyA-AI)(Sorokin *et al.*, 1986), dermal lesions clinically and histologically similar to those observed in limited scleroderma (chronic scleroderma) of man can be reproducibly elicited (Bos *et al.*, 1989). In this model, Lewis (LEW) rats are lethally irradiated, reconstituted with syngeneic bone marrow and treated for 6 weeks with CyA. After withdrawal of CyA medication an acute dermatitis develops which subsequently evolves into chronic scleroderma (Bos *et al.*, 1989; Wodzig et al., 1991). CyA-AI can be reproducibly elicited in young rats (Fischer *et al.*, 1990) or mice (Cheney *et al.*, 1985; Bryson *et al.*, 1989) and has also been observed in adult humans (Jones *et al.*, 1989; Hess

et al., 1992). Under these circumstances, CyA has been reported to prevent the re-establishment of tolerance to self and the development of CyA-AI appears to be associated with reappearance of $CD4^+$ T cells (Bos *et al.*, 1988; Fischer *et al.*, 1991) and/or the induction of cytolytic T lymphocytes (CTL's) recognizing a public MHC class II determinant (Hess *et al.*, 1985). Several studies have demonstrated an essential role for the thymus in the induction of CyA-AI (Sorokin *et al.*, 1986). Therefore, this model in terms of etiology is of (auto)immune origin, and in terms of pathogenesis manifests scleroderma-like skin lesions. In 95% of patients with SS circulating autoantibodies against nuclear or cytoplasmic constituents were detected (Bernstein *et al.*, 1982; Krieg and Meurer, 1988). Antibody titer did not show a clinical correlation with disease activity (Krieg and Meurer, 1988). Despite the fact that there was some overlap, the specificity of certain autoantibodies correlated well with the syndrome in which they occur (Guldner *et al.*, 1986). In patients suffering from SS several autoantibody specificities have been observed (Tan *et al.*, 1980). Thirty percent of patients with diffuse SS (with severe skin, cardiac, and/or pulmonary involvement) had autoantibodies to DNA-topoisomerase I (Van Venrooij *et al.*, 1985; Weiner *et al.*, 1988). In 70-80% of patients with limited scleroderma (CREST variant of scleroderma) anti-centromere/kinetochore antibodies could be detected (Tan *et al.*, 1980; Kallenberg *et al.*, 1982; Krieg *et al.*, 1988). These autoantibodies recognized the centromere proteins A, B, and C (CENP-A or CR19, 17/19 kD; CENP-B, 80 kD; and CENP-C, 140 kD)(Earnshaw *et al.*, 1986).

The present study was performed to examine by means of Western blots whether sera from rats with CyA-AI and scleroderma-like lesions contained autoantibodies akin to those listed above and if so, whether these are associated with the scleroderma-like lesions observed in CyA-AI. Indeed rats with CyA-AI were found to develop autoantibodies to nuclear and non-nuclear constituents, but the scleroderma-like lesions developed independently of the presence of such autoantibodies.

Materials and methods

Animals

Female, specific pathogen-free (SPF) Lewis (LEW, $RT1^l$) rats were obtained from our own breeding stock (Department of animal facilities, Univ. of Limburg). Animals were used as bone marrow donors and recipients at the age of 4-6 weeks. Serum samples were obtained by retro-orbital puncture.

Protocol for induction of CyA-AI

LEW rats were given 8.5 Gy at 0.5 Gy/min. using a Röntgen irradiation machine (Philips MG320, Hamburg, Germany) one day prior to syngeneic bone marrow transplanta-

tion (BMT). Marrow donor rats were killed by cervical dislocation under ether anesthesia. Bone marrow was collected from tibias and femurs in Dulbecco's balanced salt solution supplemented with 2% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) (DBSS). Recipient rats received $6x10^7$ viable syngeneic bone marrow cells in 1.0 ml DBSS intravenously into a tail vein. CyA, a gift from Sandoz Co. Ltd., Basel, Switzerland, was dissolved in olive oil at a concentration of 7.5 mg/ml. Rats received 7.5 mg/kg/day CyA subcutaneously for 6 weeks starting on the day of BMT. CyA-AI usually developed 2-3 weeks after cessation of CyA administration.

Experimental design

Twenty LEW rats were subjected to the above mentioned protocol for induction of CyA-AI and are referred to as LEW/XCyA. Control groups included: 6 LEW rats receiving X-irradiation, syngeneic bone marrow, and CyA-solvent (olive oil) for 42 days (LEW/XOil); 6 age-matched LEW treated with CyA for 42 days (cyaLEW); and 6 age-matched, non-treated LEW (nLEW). Serum samples were obtained prior to X-irradiation and syngeneic bone marrow reconstitution, 6 weeks after BMT at the time of CyA or solvent withdrawal, and 2, 4, 6, 8, 10, 12, 18, and 30 weeks after cessation of CyA or solvent administration.

Scoring of macroscopic pathology

After withdrawal of CyA, development of CyA-AI was assessed by examination of the rats twice a week for signs and symptoms of disease including red acra (hyperemia of the skin of the ears, feet and tail), inflammation of the eyelids, dermatitis, alopecia and weight loss (Wodzig *et al.*, 1991).

Preparation of nuclear and cytoplasmic extracts

Nuclear extracts from either human HeLa or rat A8/B2 (a non-functional LEW T lymphoblast clone) cells were prepared according to Habets *et al.* (1983). All procedures were carried out at 4°C unless stated otherwise. In brief, cells were harvested, pelleted by centrifugation (5 min at 800xg), washed with 130 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4 (NKM), and pelleted again. Cells were resuspended in 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4 supplemented with 0.5 mM phenylmethylsulfonyl-fluoride (PMSF)(RSB10). After addition of a mixture of 0.5% sodium deoxycholate (DOC) and 1% Tween 40, cells were homogenized by 10 strokes of a pestle. Cell nuclei were pelleted, washed once with RSB10 and resuspended in 110 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 10 mM Tris-HCl pH 7.4 (RSB110) at a density of 10^8 nuclei/ml. The nuclei were incubated with 500 µg/ml DNase I (type II) and 100 µg/ml RNase A (type III-A; both Sigma, St. Louis, USA) for 1 h at room temperature. Finally, an equal amount of sample buffer (20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 65 mM Tris-HCl pH 6.8) was added to this total nuclear protein fraction and the suspension was boiled for 3 min. Nuclear extract of $7x10^7$ cells (1.5 mg protein) was loaded onto one polyacrylamide gel.

Cytoplasmic extracts were prepared by swelling washed HeLa or A8/B2 cells in hypotonic buffer (1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM PMSF, 10 mM HEPES pH 7.9) for 20 min. Next, the cells were homogenized with a Dounce homogenizer and 0.1 volume of a hypertonic solution (1.4 M KCl, 30 mM $MgCl_2$, 0.3 mM HEPES pH 7.9) was added. The cytoplasmic extract was cleared by centrifugation for 10 min at 3,000xg and diluted by adding 1/3 volume of sample buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 125 mM Tris-HCl pH 6.8) to the pellet; the resulting solution was boiled for 3 min. Cytoplasmic extract of $4x10^7$ cells (1.5 mg protein) was loaded onto one polyacrylamide gel.

Polyacrylamide gelelectrophoresis (PAGE) and protein blotting

PAGE was performed on 1.4 mm-thick slab gels in 192 mM glycine, 25 mM Tris-HCl pH 8.3, 0.1% sodium-dodecyl-sulphate (SDS) with a 4% stacking gel and a 10% or 13% resolving gel. Proteins were loaded over the entire width of the gel and separated according to their molecular weight. After 4 h run at 40 mA per gel (limited to 75 mA/300 V) separated proteins were electrophoretically transferred onto nitrocellulose sheets (0.45 µm; Schleicher and Schuell, Germany) in 192 mM glycine, 25 mM Tris-HCl pH 8.3, 0.015% SDS and 20% methanol at 30 V/250 mA overnight at 4°C. After transfer blots were dried and stored at room temperature.

Detection of antigens

All steps were carried out at room temperature unless stated otherwise. Free protein binding sites on the nitrocellulose sheets were blocked by pre-incubation buffer (PI; 350 mM NaCl, 0.5 mM PMSF, 3% BSA, 10 mM Tris-HCl pH 7.6) for 1 h. Incubation with 1:20 diluted rat or human serum was performed overnight at 4°C in extractable nuclear antigen buffer (ENA; 150 mM NaCl, 0.1 mM PMSF, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 0.3% BSA, 10 mM Tris-HCl pH 7.6). After washing for 10 min with ENA-buffer and 2x10 min with PT (0.5% Triton X-100 in PBS) Ig or IgG immune complexes were detected by incubating the blots for 2 h with horseradish peroxidase (HRP)-conjugated rabbit anti-rat Ig (DAKO, Glostrup, Denmark) or goat anti-human IgG (Cappel/ Organon Teknika, Turnhout, Belgium) in 0.5% BSA in PT. After washing for 2x10 min with PT and 2x10 min with PBS, immune complexes on nitrocellulose sheets were visualized by incubation in HRP substrate solution (PBS containing 0.5 mg/ml 4-chloro-1-naphtol and 0.012% H_2O_2) for 10 min.

Reference sera from patients with autoimmune diseases and from BN rats with $HgCl_2$-induced autoimmunity

First, nuclear extract from HeLa cells and nuclear and cytoplasmic extracts from rat A8/B2 cells were incubated with reference patient sera containing anti-B'/B (Sm) antibodies (a marker for systemic lupus erythematosus, SLE), anti-DNA-topoisomerase I (Scl-86 or Scl-70) antibodies (a marker for diffuse systemic sclerosis), anti-centromere (CR19) antibodies (a marker for the CREST syndrome), anti-SS-B/La antibodies (predominantly found in sera from patients with SLE and Sjögren's syndrome), anti-56K antibodies (most commonly found in sera from patients with Sjögren's syndrome), and anti-ribo-

nucleoprotein (RNP) antibodies (often associated with mixed connective tissue disease and SLE) respectively. In this way autoantibodies associated with certain human autoimmune diseases, i.e. directed to Scl-86 (or Scl-70), nuclear RNP, B'/B (Sm), SS-B/La, SS-A/Ro antigens and histones can, if present, be detected in rats with CyA-AI. Furthermore, the molecular weight of constituents detected by autoantibodies from rats with CyA-AI can be estimated more precisely.

Furthermore, nuclear and cytoplasmic extracts from rat A8/B2 cells were incubated with sera from Brown Norway (BN) rats with mercuric chloride ($HgCl_2$)-induced autoimmune nephritis. Inbred, 3-4 months old BN rats, weighing 300 g received $HgCl_2$ subcutaneously at a dose of 1 mg/kg body weight at day 1, 3, 5, 7, and 9. $HgCl_2$ induces proliferation of B and $CD4^+$ T cells, hyperimmunoglobulinemia and production of autoantibodies such as anti-nuclear and anti-glomerular basement membrane (GBM) antibodies. Anti-GBM antibodies are responsible for an autoimmune glomerulonephritis resulting in proteinuria. Both anti-GBM autoantibody serum levels and proteinuria reached maximum values around day 15 and decreased gradually thereafter (Aten *et al.*, 1988). Sera from $HgCl_2$-treated BN rats were kindly donated by Dr. J.J. Weening (Dept. of Pathology, University of Amsterdam, The Netherlands).

Results

Cross-reactivity of human autoantibodies to human and rat cell antigens

In order to characterize extractable nuclear and cytoplasmic antigens in the rat in terms of known human equivalents Western blots of nuclear extract from HeLa cells and nuclear and cytoplasmic extracts from A8/B2 cells were incubated with patient sera containing anti-B'/B (Sm), anti-DNA-topoisomerase I (Scl-86 or Scl-70), anti-centromere (CR19), anti-SS-B/La, and anti-56K antibodies, respectively (Fig. 4.1). Human autoantibodies to Scl-86 (lane 10) cross-reacted with a rat nuclear antigen of the same molecular weight (lane 6). A mixture of human autoantibodies to 56K (M_w 56 kD) and La/SS-B (M_w 50 kD; lane 11) cross-reacted with rat antigens of the same molecular weights present in both nuclear (lane 8) and cytoplasmic (lane 7) fractions. Human autoantibodies to B'/B antigens (M_w 29/28 kD; lane 9) cross-reacted only with the lower molecular weight B-antigen (M_w 28 kD) present in both nuclear (lane 4) and cytoplasmic (lane 3) fractions in the rat. Furthermore, this anti-B'/B serum recognized a rat nuclear antigen (lane 4) with a molecular weight of approximately 95 kD which is not identical with the Scl-86 antigen (M_w 95 kD; lanes 6 and 10), because this serum contained no anti-Scl-86 antibodies (lane 9). Human autoantibodies to CR19 (lane 10) did not cross-react with rat nuclear and cytoplasmic antigens. Pooled (n=5) normal human sera (lanes 1 and 2) showed only a weak background staining with rat nuclear extract, except for two antigens of low molecular weight, probably histones H2A and H2B (lane 2).

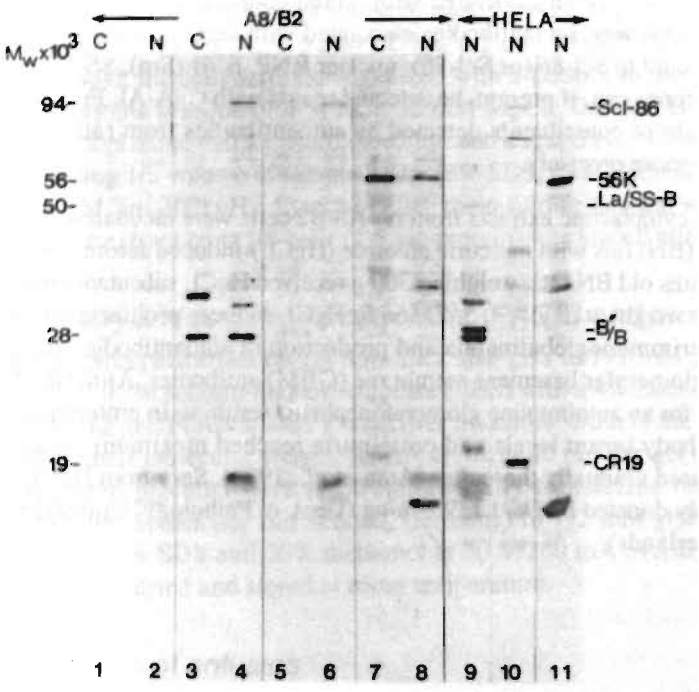
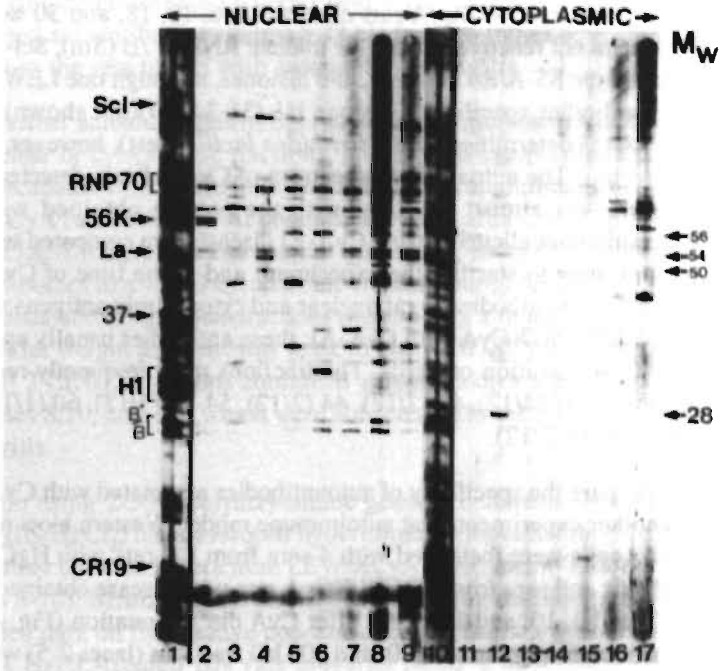


Figure 4.1: Detection of cross-reactivity of human autoantibodies to human and rat cell antigens by immunoblotting. *Nuclear (N) extracts from HeLa cells (lanes 9-11) and A8/B2 rat cells (lanes 2, 4, 6, 8) and cytoplasmic (C) extract from A8/B2 cells (lanes 1, 3, 5, 7) were electrophoresed in a 13% polyacrylamide gel, transferred onto nitrocellulose and the resulting blot was incubated with: lanes 1, 2: normal human serum; lanes 3, 4, 9: human serum containing anti-B'/B antibodies; lanes 5, 6, 10: human serum containing anti-Scl-86 and anti-CR19 antibodies; lanes 7, 8, 11: human serum containing anti-La/SS-B and anti-56K antibodies.*

Autoantibodies to human cell antigens in rats with CyA-AI

Western blots of nuclear and cytoplasmic extracts from HeLa cells were incubated with reference patient sera (see legends Figs. 4.2A and 4.2B) and sera from LEW/XCyA (n=20) obtained prior to the start of the experiment, 6 weeks after BMT at the time of CyA withdrawal, and 8 weeks after CyA withdrawal. Multiple autoantibodies to human nuclear and cytoplasmic antigens were only demonstrable at moderate titers (20-50) in sera from LEW/XCyA with manifest CyA-AI disease (9/20) but not prior or during CyA administration. In sera of LEW/XOil, cyaLEW or even nLEW controls natural autoantibodies against human nuclear and cytoplasmic antigens are demonstrable but these autoantibodies are also present in LEW/XCyA. Experimental LEW/XCyA developed autoantibodies directed to nuclear fractions with M_w's of about 35 (Fig. 4.2A, lane 6), 37 (Fig. 4.2A, lanes 6 and 7; Fig. 4.2B, lane 3), 40 (Fig. 4.2A, lanes 4 and 6), 54 (Fig. 4.2B, lane 4), 80 (Fig. 4.2A, lane 6), and 100 kD (Fig. 4.2A, lane 4). LEW/XCyA in addition developed autoantibodies to cytoplasmic fractions with M_w's of about 20 (Fig. 4.2B, lane 11), 28 (Fig. 4.2A, lane 12), 35 (Figs. 4.2A/B, lane 12), 54 (Fig. 4.2A, lane 12), and 80 kD (Fig. 4.2B, lanes 11 and 13). All sera from LEW/XCyA and LEW/XOil tested contained natural autoantibodies against a 60 kD nuclear (Fig. 4.2A, lanes 2-9; Fig. 4.2B, lanes 2-7) and cytoplasmic (Fig. 4.2B, lanes 9-15) fraction.

Figure 4.2: Detection of autoantibodies to human cell antigens in rats with CyA-AI.
A. *Nuclear (lanes 1-9) and cytoplasmic (lanes 10-17) extracts from HeLa cells were electrophoresed in a 10% polyacrylamide gel, transferred onto nitrocellulose and the resulting blot was incubated with: lane 1, 10: human reference sera (see Materials and Methods); lanes 2-7, 11-16: 6 LEW/XCyA sera from rats with CyA-AI; lanes 8, 17: serum from a LEW/XOil control; lane 9: serum from a LEW/XCyA rat with CyA-AI and myasthenia gravis.*



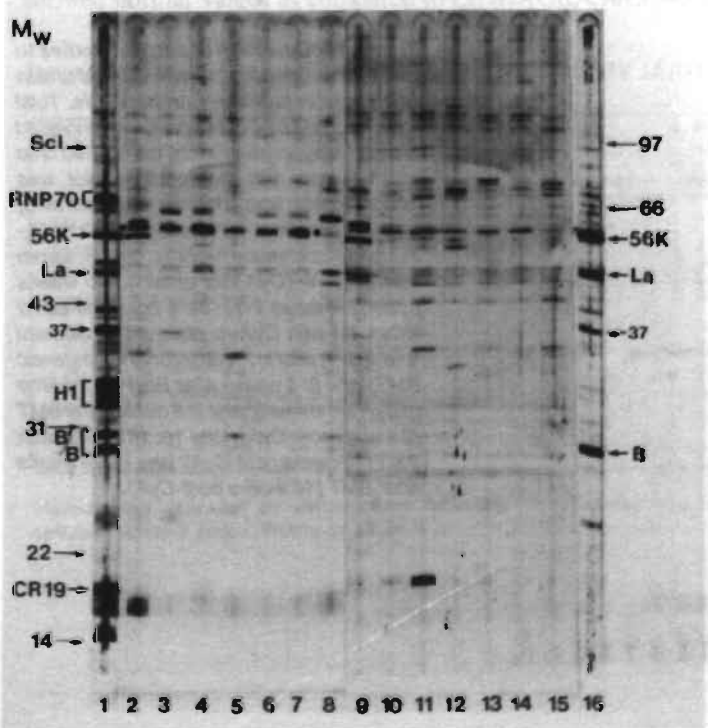


Figure 4.2: Detection of autoantibodies to human cell antigens in rats with CyA-AI.
B. *Nuclear (lanes 1-8) and cytoplasmic (lanes 9-16) extracts from HeLa cells were electrophoresed in a 10% polyacrylamide gel, transferred onto nitrocellulose and the resulting blot was incubated with: lane 1, 16: human reference sera (see Materials and Methods); lanes 3-7, 10-14: 5 LEW/XCyA sera from rats with CyA-AI; lanes 8, 15: serum from LEW/XOil control; lane 2, 9: serum from a LEW/XCyA rat with CyA-AI and myasthenia gravis.*

Autoantibodies to rat cell antigens in rats with CyA-AI

Sera from 20 LEW/XCyA rats obtained 2, 6, 10, 18, and 30 weeks after withdrawal of CyA were not reactive against the nuclear RNP, B'/B (Sm), Scl-86 (or Scl-70), SS-B/La, cytoplasmic SS-A/Ro antigens, and histones, although one LEW/XCyA serum contained autoantibodies specific for histone H1 (31-34 kD)(not shown). Antibodies directed to dsDNA (as determined by the *Crithidea lucillae* test), however, could not be detected in this serum. The autoantibody spectrum of CyA-AI rats detected with human HeLa cell extracts was almost identical with the pattern obtained with rat A8/B2 extracts. Autoantibodies elicited during CyA-AI disease were compared with autoantibody profiles present prior to starting the experiment and at the time of CyA withdrawal. Multiple specific autoantibodies to rat nuclear and cytoplasmic antigens were demonstrable in 12 out of 20 LEW/XCyA with CyA-AI; these antibodies usually appeared from 8-10 weeks after CyA cessation onwards. The fractions most frequently recognized had molecular weights of 37 (4/12), 40 (2/12), 44 (2/12), 54-56 (5/12), 60 (1/12), 70 (1/12), 80 (2/12), and 100 kD (2/12).

To compare the specificity of autoantibodies associated with CyA-AI with those elicited in another experimental rat autoimmune model, Western blots of total cell extracts from A8/B2 cells were incubated with 4 sera from BN rats with $HgCl_2$-induced autoimmune nephritis and sera from 2 LEW/XCyA rats with disease obtained on 0 and 6 weeks post-BMT and 2, 10, and 18 weeks after CyA discontinuation (Fig. 4.3). BN sera contained autoantibodies against 44, 80, and 100 kD fractions (lanes 2-5) which were shared by sera of LEW/XCyA rats obtained on 10 (lanes 6, 10) and 18 weeks (lane 11) after cessation of CyA. In LEW/XCyA rats, these autoantibodies mainly developed late in the natural

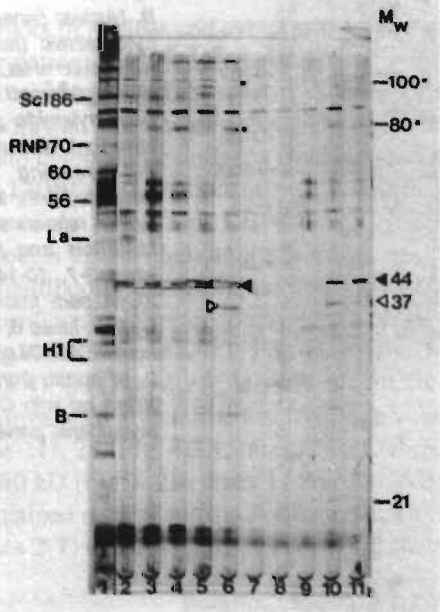


Figure 4.3 :Detection of autoantibodies to rat cell fractions in rats with CyA-AI and in BN rats with autoimmune nephritis. *Total extracts from A8/B2 cells were electrophoresed in a 10% polyacrylamide gel, transferred onto nitrocellulose and the resulting blot was incubated with: lane 1: human reference sera; lane 2-5: sera from 4 different rats with $HgCl_2$-induced autoimmune nephritis; lane 6: serum from a LEW/XCyA rats obtained 10 weeks post-CyA; lanes 7-11: sera from one LEW/XCyA rat with CyA-AI obtained at different time; lane 7: prior to X-irradiation and syngeneic BMT; lane 8: 6 weeks after BMT (at the time of CyA withdrawal); lane 9: 8 weeks after BMT (2 weeks post-CyA); lane 10: 16 weeks after BMT (10 weeks post-CyA); lane 11: 24 weeks after BMT (18 weeks post-CyA).*

history of CyA-AI and could neither be detected prior to (lane 7) and during CyA administration (lane 8), nor during acute disease (2 weeks post-CyA)(lane 9). Sera from the LEW/XCyA rats contained autoantibodies against a 37 kD fraction (lanes 6, 9, and 10), which were not detected in the sera of rats with autoimmune nephritis.

In order to determine whether autoantibodies in BN rats with autoimmune nephritis specifically recognized nuclear or cytoplasmic fractions, nuclear and cytoplasmic extracts from A8/B2 cells were incubated with serum from one BN rat with autoimmune nephritis and sera from 3 LEW/XCyA rats with CyA-AI obtained 10 weeks after cessation of CyA (Fig. 4.4). Two of these LEW/XCyA rats and the rat with $HgCl_2$-induced autoimmune nephritis shared autoantibodies to a 44 kD cytoplasmic fraction (lanes 6,8, and 10), and to 80 kD and 100 kD nuclear and cytoplasmic fractions (lanes 5-10), although the staining of these two high molecular weight antigens was weak in one LEW/XCyA serum (lanes 9 and 10). Sera from all 3 LEW/XCyA rats contained autoantibodies against a 37 kD cytoplasmic fraction (lanes 8,10, and 12), which were not detected in the serum of the rat with autoimmune nephritis.

Separation of total serum using 2-30% polyacrylamide gelelectrophoresis showed that $HgCl_2$-treated BN rats (BN/$HgCl_2$) had developed hyperimmunoglobulinemia (Fig. 4.5; lanes 1-3) which was neither detected in sera from LEW/XCyA rats (lanes 4-7) nor in sera from control rats (lanes 8-10). Serum protein electrophoresis using cellulose acetate as support medium provided data on the relative concentrations of immunoglobulins and again demonstrated in $HgCl_2$-treated BN rats elevated relative concentrations of γ-, β-, and α1-globulins as compared to nBN and nLEW. In contrast, LEW/XCyA rats showed normal values as compared to LEW/XOil, cyaLEW, and nLEW (Table 4.1).

***Table 4.1:** Fraction of total serum of $HgCl_2$-treated BN,normal BN, LEW/XCyA and control LEW rats.*

Rat strain	Relative fraction of total serum (%)					
	Gamma	Beta	Alpha2	Alpha1	Albumin	A/G ratio[a]
BN/$HgCl_2$	7.3	28.1	4.5	15.2	44.9	0.8
BN/$HgCl_2$	5.1	23.7	7.1	15.7	48.3	0.9
nBN	1.5	13.0	6.1	6.9	72.5	2.6
nBN	2.4	16.0	7.3	6.2	68.1	2.1
LEW/XCyA	2.7	15.0	2.3	5.6	74.5	2.9
LEW/XCyA	2.8	17.1	3.3	4.9	71.8	2.6
LEW/XOil	2.3	15.5	2.7	5.3	74.1	2.9
cyaLEW	1.9	14.5	2.2	5.8	75.7	3.1
nLEW	3.0	12.8	2.0	5.8	76.3	3.2

Values were assessed by densitometer scanning of separated and visualized protein bands on cellulose acetate strips. [a]Ratio of albumin to total globulin fraction.

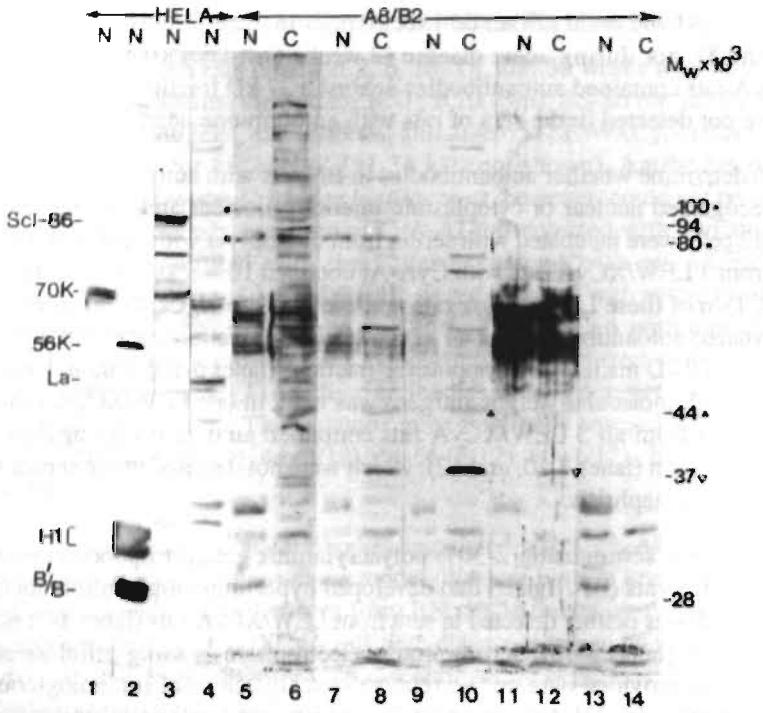


Figure 4.4: Detection of autoantibodies to rat cell fractions in rats with CyA-AI and in a BN rat with autoimmune nephritis. *Nuclear (N) extracts from HeLa cells (lanes 1-4) and A8/B2 cells (lanes 5, 7, 9, 11, 13) and cytoplasmic (C) extract from A8/B2 cells (lanes 6, 8, 10 ,12, 14) were electrophoresed in a 10% polyacrylamide gel, transferred onto nitrocellulose and the resulting blot was incubated with: lane 1: human serum containing anti-RNP antibodies; lane 2: human serum containing anti-56K, anti-histone H1 and anti-B'/B antibodies; lane 3: human serum containing anti-Scl-86 and anti-CR19 antibodies; lane 4: human serum containing anti-La/SS-B antibodies; lanes 5, 6: serum from a rat with $HgCl_2$-induced autoimmune nephritis; lanes 7, 8, lanes 9, 10, and lanes 11, 12: serum from 3 different LEW/XCyA rats with CyA-AI; lanes 13, 14: normal LEW rat serum.*

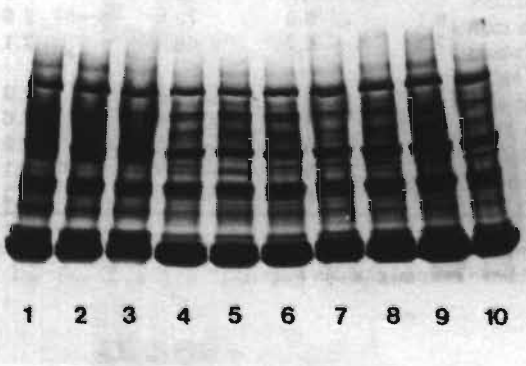


Figure 4.5: Gradient 2-30% gel electrophoresis of total serum from LEW and BN rats.
Lane 1-3: 3 different sera from BN/$HgCl_2$ rats; lane 4-7: 4 different sera from LEW/XCyA rats with CyA-AI; lane 8: serum from a LEW/XOil rat; lane 9: serum from a nLEW rat; and lane 10: serum from a cyaLEW rat.

Discussion

This study shows that autoantibodies to extractable nuclear and cytoplasmic fractions are elicited in rats with CyA-AI. Furthermore, it was demonstrated that these autoantibodies are not required for, nor pathological related to the development of chronic scleroderma-like lesions. Finally, the specificity of some of the autoantibodies detected in LEW rats with CyA-AI disease also occurred in BN rats with $HgCl_2$-induced autoimmune glomerulonephritis, suggesting that analogous autoimmune mechanisms are involved.

Crucial to answering the question whether or not autoantibodies considered of clinical diagnostic importance in man are associated with chronic scleroderma-like lesions in the rat model of CyA-AI is the observation that human autoantibodies to 95 kD (Scl-86), 56 kD, SS-B/La, and B'/B (Sm) cross-reacted with rat nuclear and cytoplasmic antigens (Fig. 4.1). However, no such rat autoantibodies were observed in rats with CyA-AI. The molecular weight of Scl-86/topoisomerase I has been reported to be 67 to 100 kD (Scl-70 or Scl-86 antigen); enzyme molecules of various M_w's ranging from 70 to 100 kD have been found to be functionally active (Hildebrandt *et al.*, 1990). The antigen present in HeLa and A8/B2 cells was identified as a 95 kD protein provided protease inhibitors were included in the extraction buffer (Guldner *et al.*, 1986). Although human autoantibodies to the CR19 antigen (associated with CREST syndrome) extractable from HeLa cells do not recognize a 18-20 kD antigen in the rat, such antibodies may recognize a 50 kd peptide in rat nuclear extracts (Kremer *et al.*, 1988). In this study, however, antibodies to this 50 kD peptide were not observed. One rat with CyA-AI and autoantibodies to histone H1 (31-34 kD) excepted, none of the rats with chronic CyA-AI showed autoantibodies comparable to those with diagnostic importance in human immune-mediated "connective tissue" diseases. Given the similarity of the skin lesions of chronic CyA-AI to those of scleroderma in man (Bos *et al.*, 1989), these observations support the clinical observation that autoantibodies to the Scl-86 or CREST antigen are not causatively related to the pathogenesis of the lesions. In the rat model of CyA-AI antibodies of pathogenetic significance may be elicited, e.g. to rat acetylcholine receptor with ensuing myasthenia gravis (Verschuuren *et al.*, 1989). In this respect the model is reminiscent of the myasthenia gravis occasionally seen also after bone marrow transplantation in man (Bolger *et al.*, 1986; Grau *et al.*, 1990; Melms *et al.*, 1992). On the other hand, the model is clearly different from the CyA-AI disease models in certain mouse strains which are associated with autoimmune antibody-mediated endocrinopathies (Sakaguchi and Sakaguchi, 1988, 1989).

Hyperactivity of the immune system, especially of the B-cell compartment, resulting in polyclonal hypergammaglobulinemia is a constant feature of human and experimental autoimmunity (Marcos *et al.*, 1986). Sakaguchi *et al.* (1989) showed that CyA administration to newborn BALB/c mice resulted in organ-specific autoimmune disease in multiple endocrine organs (thyroid, stomach, ovaries, or testes) with development of autoantibodies specific for gastric parietal cells and oocytes as assessed by indirect

immunofluorescence. The incidence of gastritis and oophoritis was enhanced by removal of the thymus immediately after neonatal CyA treatment. Other autoimmune diseases developed in the CyA-treated and thymectomized mice as well: thyroiditis, insulitis, adrenalitis, sialoadenitis, and orchitis. These autoimmune diseases were accompanied by the appearance of serum autoantibodies specific for thyroglobulins, cell surface antigens of the Langerhans islet cells, adrenocortical cells, acinar cells of the salivary glands, and sperm respectively (Sakaguchi and Sakaguchi, 1989).

Administration of CyA after syngeneic bone marrow transplantation inhibited the development of both mature $CD4^+$ and $CD8^+$ single positive thymocytes in rodents, whereas the relative numbers of immature double positive and double negative cells were increased (Fischer *et al.*, 1991). These effects on the thymus were also reflected by changes in the periphery with fewer $CD4^+$ T cells and a reduced CD4/CD8 ratio in X-irradiated and CyA-treated animals (Bos *et al.*, 1988; Fischer *et al.*, 1991). On the other hand, elevated relative numbers of cells expressing both CD4 and CD8 molecules were detected in the peripheral blood suggesting that these cells were prematurely released from the thymus (Hess *et al.*, 1985). In the absence of counteractive regulatory T cells, these cells might differentiate into autoimmune effector T cells. The latter cells might be $CD4^+CD8^-$ and destroy target organs by conducting cell-mediated immune reactions (i.e. effecting the scleroderma-like lesions) and/or promote activation of autoantibody-forming B cells (Sakaguchi and Sakaguchi, 1990).

The next observation of interest is the fact that the majority of sera from LEW rats with CyA-AI contained autoantibodies to a variety of human and rat nuclear and cytoplasmic fractions which were shared in part (Figs. 4.3 and 4.4) with autoantibodies present in sera from BN rats with $HgCl_2$-induced autoimmune nephritis. $HgCl_2$ administration elicits in BN rats a chronic but transient graft-versus-host disease associated with hypergammaglobulinemia and autoantibody-mediated glomerular basement membrane damage (Tournade *et al.*, 1990; Goldman *et al.*, 1991). In contrast to CyA-AI pathogenesis is not influenced by thymectomy (Pelletier *et al.*, 1988a), and requires the presence of BN major and minor histocompatibility antigens (Pelletier *et al.*, 1986, 1988b). Furthermore, this disease is associated with induction of autoreactive $CD4^+$ T cells against MHC class II antigens which cause - in an unknown fashion - B cell triggering (Pelletier *et al.*, 1986, 1988b). The self limitation observed in this model is associated with both a decrease in the frequency of anti-MHC class II T cells and the emergence of $CD8^+$ T cells able to suppress these autoreactive T cells (Pelletier *et al.*, 1990). CyA treatment during $HgCl_2$ administration was found to prevent the development of autoimmune reactivity and to induce a prolonged unresponsiveness (might be due to direct functional deletion of autoreactive T lymphocytes) to $HgCl_2$, which is not mediated by active immune suppression (Aten *et al.*, 1988). $HgCl_2$ administered to LEW rats on the other hand causes no disease but non-antigen specific $CD8^+$ T cell-dependent immune suppression (Pelletier *et al.*, 1987); depletion of $CD8^+$ T cells abrogated immunosuppression, however, did not allow the disease to occur in this strain (Pelletier *et al.*, 1990). LEW rats could differ from BN rats in their response to $HgCl_2$ either because they do not develop autoreactive T

helper cells and/or because suppressor cells are preferentially induced preventing polyclonal activation of B cells (Pelletier *et al.*, 1987).

The division of $CD4^+$ T cells into distinct subsets [T helper cell 1 (T_H1) and 2 (T_H2)], based upon cytokine production, might be a clue to explain differences between the immune response of BN and LEW rats. T_H1-like cells produce IL-2 and IFNγ upon stimulation and provide cell-mediated immune responses (delayed type hypersensitivity, DTH), whereas T_H2-like cells produce IL-4, IL-5, IL-6, and IL-10 upon stimulation and are responsible for the provision of B cell help and induction of suppression of cell-mediated immune responses (Mosmann and Moore, 1991). Antagonistic effects between T_H1 and T_H2 subsets have been described and depend on the production of IFNγ- and IL-10 (Dubey *et al.*, 1991). BN rats showed a predominance of the T_H2 subset over T_H1; $HgCl_2$ might induce an imbalance between T_H1 and T_H2 and the lymphokines produced by T_H2-like cells may prevent the development of T_H1-type responses (Goldman *et al.*, 1991). In contrast, LEW rats showed a preponderance of the T_H1-like subset, but CyA-AI impaired T_H1-dependent (IFNγ) DTH reactions (this thesis chapter 2; Wodzig *et al.*, 1993). Therefore, in the CyA-AI model administration of CyA might suppress specific T_H1-like lymphokines (IFNγ and IL-2) causing the balance between T_H1 and T_H2 to shift towards specific T_H2-type responses (mediated by IL-4 and IL-10) and consequently (auto)antibody production. It is well known that this bias in T_H1 and T_H2 upon activation could determine the direction of the immune response (Goldman *et al.*, 1991). Indeed, studies investigating *in vitro* responses of mice with chronic GVHD have demonstrated a selective loss of T_H1 functions like production of IL-2 and IFNγ, but elevated T_H2-dependent IL-4 production. In these animals, like in the CyA-AI model, $CD8^+$ T cell functions remained intact (Hess *et al.*, 1985; De Wit *et al.*, 1993). This selective deficiency of T_H1 is associated with hyperactivation of B cell functions leading to production of autoantibodies, and hypergammaglobulinemia (De Wit *et al.*, 1993). The common denominator in the CyA-AI disease of LEW [to which the BN strain is resistant (this thesis chapter 2; Wodzig *et al.*, 1993)] and $HgCl_2$-induced disease in BN [to which LEW is resistant (Pelletier *et al.*, 1987; Dubey *et al.*, 1991)] appears the presence of GHV-like disease and anti-MHC class II T cell responses (Hess *et al.*, 1985; Sorokin *et al.*, 1986; Pelletier *et al.*, 1986, 1988b).

Hence, in these models of rodent disease autoantibodies to restricted nuclear and cytoplasmic fractions appear to be a sign of an ongoing "autoimmune" graft-versus-host disease brought about by apparently widely differing inducing protocols and thymus dependency of disease. The observation that procainamide induced SLE-like syndrome in man associated with autoantibodies to histone (Portanova *et al.*, 1982; Tortoritis *et al.*, 1988) which is also observed in CyA-AI suggests that elucidation of the mechanism(s) involved in CyA-AI may be pertinent to the pathogenesis of some autoantibody-mediated, natural or drug-induced, diseases of man.

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Chapter 5

On the localization of effector cells in Cyclosporine A - induced autoimmunity

On the localization of effector cells in Cyclosporine A - induced autoimmunity

Abstract

Lethally irradiated rats, reconstituted with syngeneic bone marrow and given Cyclosporine A (CyA) for 6 weeks, developed disease resembling allogeneic graft-versus-host disease 2 weeks after withdrawal of CyA. Other studies have demonstrated the pivotal role of the thymus in the etiology of this CyA-induced autoimmune disease (CyA-AI). In this study the question was addressed whether inducer/effector cells of CyA-AI are generated in the thymus during or after CyA administration; whether these cells stay in the thymus or, if they don't, whether they home to the secondary lymphoid organs.

Adoptive transfer of thymocytes from donors treated for induction of CyA-AI obtained one and 14 days after cessation of CyA administration did not elicit CyA-AI in irradiated secondary recipients. Furthermore, adult thymectomy of rats immediately after the course of CyA did not influence the kinetics of development of skin pathology, although weight loss commenced later in thymectomized than in sham-thymectomized rats.

Lymph node and spleen cells obtained from donors treated for induction of CyA-AI one and 14 days after withdrawal of CyA caused CyA-AI upon adoptive transfer to secondary recipients, but the symptoms of acute disease (dermatitis, alopecia and weight loss) were strikingly less severe upon transfer of lymphoid cells obtained one day after stopping CyA than 14 days thereafter. Therefore, this study demonstrates that CyA-AI inducer/effector cells are generated in the thymus during the administration of CyA. These cells exit from the thymus during CyA administration; either they home predominantly peripherally (i.e. in the skin) rather than in the secondary lymphoid organs, or they leave the thymus as inducer cells which home in the lymphoid organs where they subsequently may trigger potentially autoreactive lymphocytes as probably also present in normal individuals, or both pathways may be operative.

Introduction

Cyclosporine A (CyA), a fungal metabolite with potent immunosuppressive properties, is effective in preventing graft-versus-host disease (GVHD) secondary to allogeneic bone marrow transplantation (BMT), and in inhibiting progression of several autoimmune diseases (Tutschka *et al.*, 1979; Von Graffenried *et al.*, 1989; Bacigalupo *et al.*, 1990). Paradoxically, under certain circumstances CyA may also induce autoimmune disease. Lethally irradiated rats reconstituted with syngeneic or autologous bone marrow and given CyA for some weeks following BMT develop, after withdrawal of CyA, symptoms of disease. These symptoms, as well as the histologic lesions, are similar to those seen in acute graft-versus-host disease (GVHD) after allogeneic BMT (Glazier *et al.*, 1983a). This syngeneic GVHD (sGVHD) (Glazier *et al.*, 1983a), CyA-induced autoimmune disease (CyA-AI) (Sorokin *et al.*, 1986) or BMT-associated immune disease (BMT-ID) (Bos *et al.*, 1990) can be elicited reproducibly in young rats (Fischer and Hess, 1990) or mice (Cheney and Sprent, 1985; Bryson *et al.*, 1989) and has also been observed in adult humans (Jones *et al.*, 1989).

Several studies have demonstrated an essential role for the thymus in the induction of CyA-AI. As shown by Glazier *et al.*, shielding of the thymus during total-body irradiation prevents the induction of CyA-AI (Glazier *et al.*, 1983b). Furthermore, CyA-AI could not be elicited in animals thymectomized prior to BMT (Sorokin *et al.*, 1986).Two hypotheses prevail with respect to the role of the thymus in the kinetics of the generation of autoreactive T cells in CyA-AI. According to the first, inducer and/or effector T lymphocytes are generated *during* CyA therapy but remain ineffective due to the immunosuppressive activity of CyA (Wilson, 1989). The second hypothesis supposes that the generation of autoaggressive T lymphocytes takes place only *after* withdrawal of CyA and the ensuing decline of CyA levels in the hosts (Majoor *et al.*, 1991). The onset of CyA-AI is associated with the reappearance of $CD4^+$ T cells in the peripheral blood about two weeks after cessation of CyA administration (Bos *et al.*, 1988a; Fischer *et al.*, 1991).

Data reported for mice indicated that thymocytes obtained immediately after a 6 weeks course of CyA from either lethally irradiated and reconstituted mice or from normal mice, were effective in adoptively transferring CyA-AI to secondary syngeneic recipients (Cheney and Sprent, 1985). Although these data suggest the presence of CyA-AI effector cells or their inducers in the thymus of CyA-treated mice, we failed to reproduce this result in a preliminary experiment using non-irradiated, CyA-treated donor rats. Since adoptive transfer of CyA-AI by thymocytes has only been described for the rat using thymocytes harvested 30 days after withdrawal of CyA (Beschorner *et al.*, 1988a), an attempt was made to investigate in the rat model whether CyA-AI effector- or inducer cells are present in the thymus and the lymph nodes at the time of cessation of CyA administration. Both adoptive cell transfer studies and adult thymectomies were performed to address this question experimentally.

The results of the present study demonstrate that CyA-AI inducer/effector cells are generated and leave the thymus during the course of CyA. Adoptive transfer studies showed that auto-aggressive cells are not likely to home preferentially in the lymph nodes.

Materials and methods

Animals

Female, specific pathogen-free Lewis (LEW, $RT1^l$) rats were used as donors and recipients. Rats were obtained from our own breeding stock. Animals were used at the age of 4-6 weeks.

Protocol for induction of CyA-AI

Rats were lethally irradiated one day prior to syngeneic BMT. CyA was administered for 6 weeks starting on the day of BMT. This procedure is referred to as the standard protocol. CyA-AI usually developed 2-3 weeks after cessation of CyA administration (Bos *et al.*, 1988a).

Irradiation

Rats were given 8.5 Gy at 0.5 Gy/min. using a Röntgen irradiation machine (Philips MG320, Hamburg, Germany).

Bone marrow transplantation

Donor rats were killed by cervical dislocation under ether anesthesia. Bone marrow was collected from tibias and femurs in Dulbecco's balanced salt solution supplemented with 2% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) (DBSS). Recipient rats received $6x10^7$ viable syngeneic bone marrow cells in 1.0 ml DBSS intravenously into a tail vein.

Cyclosporine A

CyA, a gift from Sandoz Co. Ltd., Basel, Switzerland, was dissolved in olive oil at a concentration of 7.5 mg/ml. Rats were weighed daily and received 7.5 mg/kg/day subcutaneously from the day of BMT.

Scoring of macroscopic pathology

After withdrawal of CyA, development of CyA-AI was assessed by examination of the rats 1-2 times a week for signs and symptoms of disease: red acra (hyperemia of the skin of the ears, feet and tail), inflammation of the eyelids, dermatitis, and alopecia (Table 5.1)

***Table 5.1:** Coding of macroscopic pathology in CyA - AI.*

Code	Pathology
-	No pathology
E	Erythroderma of the acra (hyperemia of the skin of the ears, feet and tail)
D	Dermatitis, usually starting around the eyes and with brown/red discoloring of the fur of the neck, front and/or hind legs. Expansion is initially restricted to the ventral part of the body.
A_v	Ventral alopecia
A_g	Generalized alopecia
T	Thin fur, usually dorsal (consequence of slowly progressive disease or recovery from alopecia)

and the weights of the rats were determined (Bos *et al.*, 1989). The alopecia is akin to that described to occur in stable chimeras after allogeneic bone marrow transplantation without additional CyA therapy, although in this allogeneic model alopecia developed later than in the model of CyA-AI (Beschorner *et al.*, 1982; Bos *et al.*, 1988b).

Adoptive cell transfers

Thymocytes, mesenterial and cervical lymph node cells, and splenocytes were harvested from animals one or 14 days after withdrawal of CyA. Animals taken 14 days after cessation of CyA manifested early signs of CyA-AI. Single cell suspensions were made by passing organ fragments through nylon gauze screens. Cells were collected in DBSS, washed, and their viability assessed by trypan blue exclusion. Putative effector cells were infused together with syngeneic bone marrow cells ($6x10^7$) into 8.5 Gy irradiated secondary recipients. Secondary recipients received either 10^8 thymocytes or $3x10^7$ lymph node cells, which were in one experiment partly replaced by spleen cells.

Thymectomy

Lewis rats treated according to the protocol for induction of CyA-AI were thymectomized (Tx) or sham-thymectomized (sham-Tx) on day one after cessation of CyA administration.

Statistics

Weight differences between groups of rats were tested for statistical significance by Wilcoxon's rank sum test.

Results

Development of CyA-AI in donor rats

A 'primary' cohort of 12 rats received lethal irradiation, syngeneic BMT, and CyA for 42 days. Five out of these 12 rats were sacrificed one day after stopping CyA for adoptive transfer of either thymocytes or lymph node cells to irradiated secondary recipients. Two weeks after cessation of CyA the remaining 7 rats showed 2-22 % decrease in body weight as compared to their weights on day 6 after CyA withdrawal (Fig. 5.1). In contrast, a control group derived from another experiment consisting of 7 irradiated, bone marrow-reconstituted rats given the CyA-solvent only, showed on day 14 after cessation of injections a mean increase in body weight of 6 g (Range: 3-11 g; 2-6%). Three out of the 7 rats given CyA exhibited severe skin pathology like dermatitis, and ventral alopecia on day 14 after CyA withdrawal; the other 4 rats showed symptoms associated with the

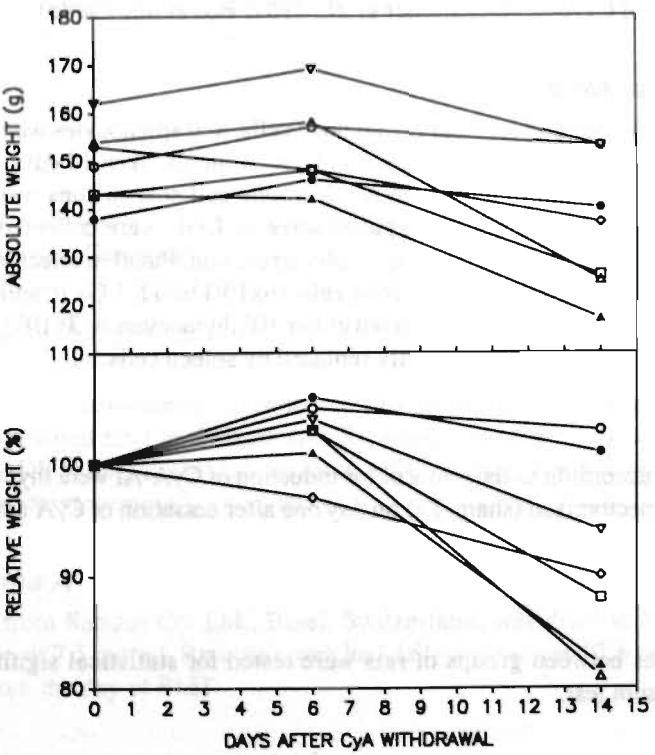

Figure 5.1: Absolute (upper panel) and relative weights (lower panel) of 7 rats treated for induction of CyA-AI according to the standard protocol, after CyA withdrawal. All 7 rats manifested symptoms of CyA-AI on day 13 after CyA withdrawal. Five additional rats from the same cohort were used for day 1 transfer of lymphoid cells.

onset of CyA-AI like erythroderma of the acra and dermatitis (Table 5.2, Group I). Therefore, also donors used one day after stopping CyA were accepted to be treated adequately for development of CyA-AI.

Transfer of thymocytes

To investigate whether autoreactive lymphocytes were present in the thymus at the time of cessation of CyA administration, pooled thymocytes obtained one day after withdrawal of CyA from 4 donors were transferred to 5 lethally irradiated secondary recipients along with normal syngeneic bone-marrow cells (Group II). For comparison, pooled thymocytes obtained 2 weeks after CyA withdrawal from 3 donors were transferred to 3 secondary recipients (Group III). The average yield of viable thymocytes per rat from donors taken one day after cessation of CyA was $5.8x10^8$ and that from rats sacrified 14 days after stopping CyA $1.3x10^8$;a reduction of 78%.

Adoptive transfer of 10^8 thymocytes, irrespective whether harvested one day or 14 days after CyA withdrawal, into secondary recipients elicited no manifestation of CyA-AI for over 100 days. In contrast, transfer of 3 x 10^7 lymph node cells and splenocytes, obtained

***Table 5.2:** Development of autoimmune skin pathology.*

Group (number of rats)	Treatment	AT or CW[a]	Skin pathology[b] on day n after AT or CW							
			0	7	10	13	21	28	35	42-63
I (7)	Standard protocol[c]; donors for "day 14" transfers	CW	7x-	6x- 1xD	2x- 1xE 4xD	4xD 3xA_v	(rats sacrified on day 14)			
IV (5)	Recipients of "day 1" LNC	AT	5x-	5x-	5x-	5x-	5xE	1x- 4xD	5xD	5xT
V (3)	Recipients of "day 14" LNC/SC	AT	3x-	3x-	1x- 2xD	3xD	3xD	3xA_v	3xA_v	3xT
VII (4)	Standard protocol; sham-Tx day 0	CW	4x-	4x-	ND[d]	1x- 3xD	1x- 3xA_v	1x- 3xA_v	1x- 3xA_v	3xA_v 1xT
VIII (5)	Standard protocol; Tx day 0	CW	5x-	5x-	ND	1x- 4xD	3xD 2xA_v	3xD 2xA_v	1xD 4xA_v	1xD 4xA_v
IX (30)	Standard protocol	CW	39x-	39x-	ND	26x- 4xE 4xD 5xA_v	18x- 2xE 10xD 8xA_v 1xA_g	ND	9x- 21xA_v 9xA_g	4x- 3xD 18xA_v 10xA_g 4xT

[a]AT, adoptive transfer; CW, CyA withdrawal. [b]Scoring of skin pathology: see Table 5.1. [c]Standard protocol: Lethal X-irradiation, BMT, and CyA for 42 days. [d]ND: No data available.

14 days after withdrawal of CyA, to lethally irradiated and reconstituted secondary recipients evoked CyA-AI as assessed by development of dermatitis and alopecia (Table 5.2, Group V) and concomitant weight loss within 2 weeks after cell transfer (Fig. 5.2). Differences in relative weights between combined thymocyte recipients and spleen- and lymph node cell recipients were significant on days 14-42 after adoptive transfer (day 14-35: $P < 0.02$; day 42: $P = 0.05$).

Effect of thymectomy on development of CyA-AI

In a different aproach to the same question, 9 lethally irradiated rats reconstituted with syngeneic bone marrow and given CyA for 42 days, were thymectomized (n=5) or sham-thymectomized (n=4) one day after stopping CyA. Symptoms of CyA-AI developed by the same kinetics in both groups (Table 5.2, Groups VII and VIII). However, the weight loss usually associated with acute CyA-AI occurred two weeks later in Tx rats than in sham-Tx rats. The relative weights of Tx and sham-Tx rats differed significantly on days 7 and

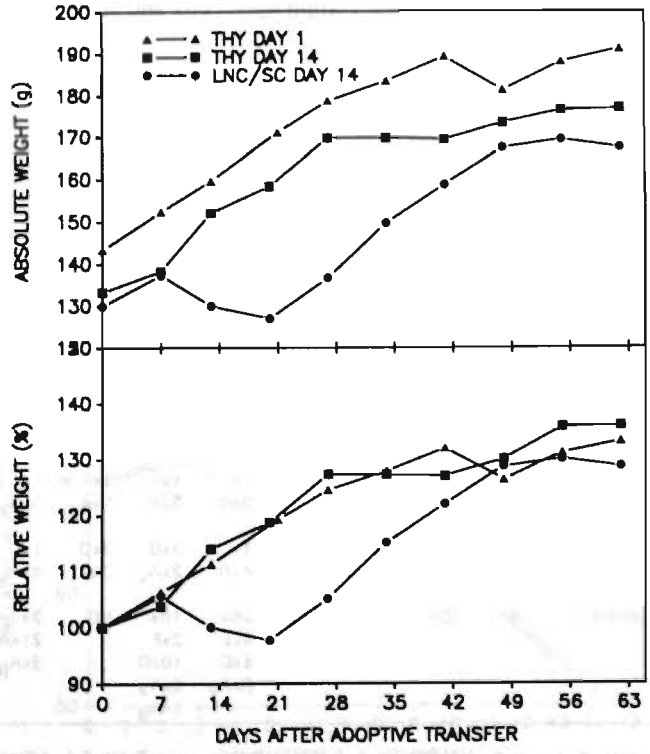

Figure 5.2: Effect of adoptive transfer of 10^8 thymocytes obtained from rats treated for induction of CyA-AI on day 1 (▲) and day 14 (■) after CyA withdrawal, on the weight of secondary recipients. For comparison, the effect of transfer of $3x10^7$ lymph node and spleen cells obtained 14 days after withdrawal of CyA is shown (●). Upper panel, absolute weights; lower panel, relative weights.

14 after CyA withdrawal ($P < 0.03$) (Fig. 5.3). Kinetics of development of CyA-AI in sham-Tx rats were comparable to that in a control group derived from a separate experiment consisting of 39 female LEW rats that were lethally irradiated, reconstituted with syngeneic bone-marrow, and given CyA for 35 days (Table 5.2, Group IX), although the relative weights of both groups were significantly different on day 7 after CyA withdrawal. Onward from day 28 post-CyA withdrawal the relative body weights of the three groups of rats that received CyA were not significantly different (Fig. 5.3).

Transfer of lymph node cells and spleen cells

The average yield of viable lymph node cells from rats sacrificed one day after stopping CyA was $6x10^7$, and that of spleen cells $12.5x10^7$ per animal. For rats sacrified 2 weeks after CyA withdrawal these figures were $2.9x10^7$ (=48%) and $4.5x10^7$ (=38%) respectively. Therefore, in the adoptive transfer performed 2 weeks after CyA withdrawal lymph node cells were supplemented with spleen cells to match the number of lymphoid cells

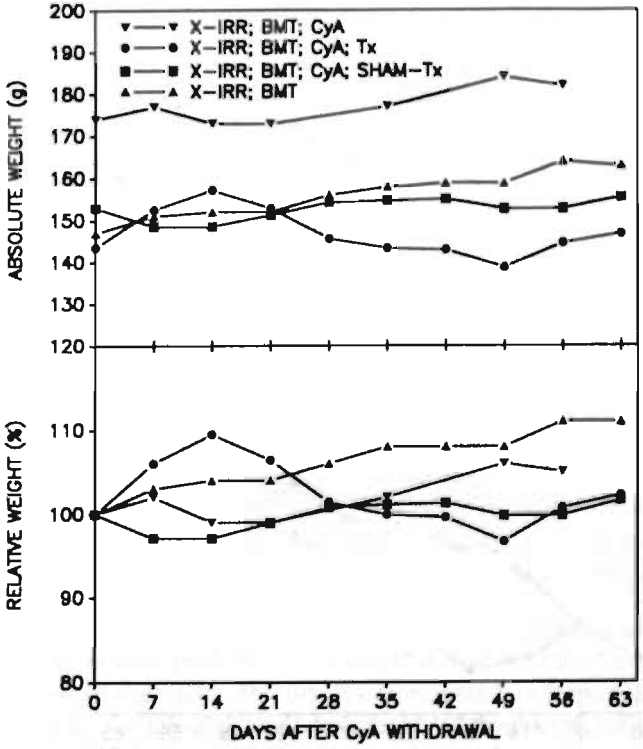

Figure 5.3: Effect of Tx (●) and sham-Tx (■) of rats treated for induction of CyA-AI on their weights (upper panel, absolute weights; lower panel, relative weights). Thymectomies were performed one day after CyA withdrawal. For comparison, from separate experiments the weight curves of 39 non-operated rats treated for induction of CyA-AI (▼), and of 6 rats given X-irradiation, BMT, and the CyA-solvent only (▲) are shown.

transferred one day after stopping CyA. Transfer of this mixture of lymph node and spleen cells (2.75:1) obtained 14 days after CyA withdrawal from 3 donor rats exhibiting CyA-AI, to 3 irradiated and reconstituted secondary recipients readily elicited CyA-AI. Two weeks after cell transfer recipients developed symptoms of CyA-AI and showed a 4-7 % decrease in body weight as compared to day 7. In contrast, there was no reduction in the weights of 5 rats that received lymph node cells obtained one day after cessation of CyA administration. This difference was significant between 14 and 28 days after adoptive cell transfer ($P < 0.04$) (Fig. 5.4). The symptoms of CyA-AI developed 2 weeks later and were less vehement in rats that received lymph node cells obtained from donors one day after cessation of CyA than in recipients of lymphoid cells harvested 14 days after CyA withdrawal. Ultimately, in rats from both groups acute pathology evolved to a "chronic", mild variety onward from day 42 after cell transfer (Table 5.2, Group IV and V).

Furthermore, an attempt was made to transfer CyA-AI to secondary recipients using donors that received CyA for 6 weeks, but without the preceding lethal X-irradiation and BMT (Group VI). Neither thymocytes (data not shown) nor pooled lymph node cells

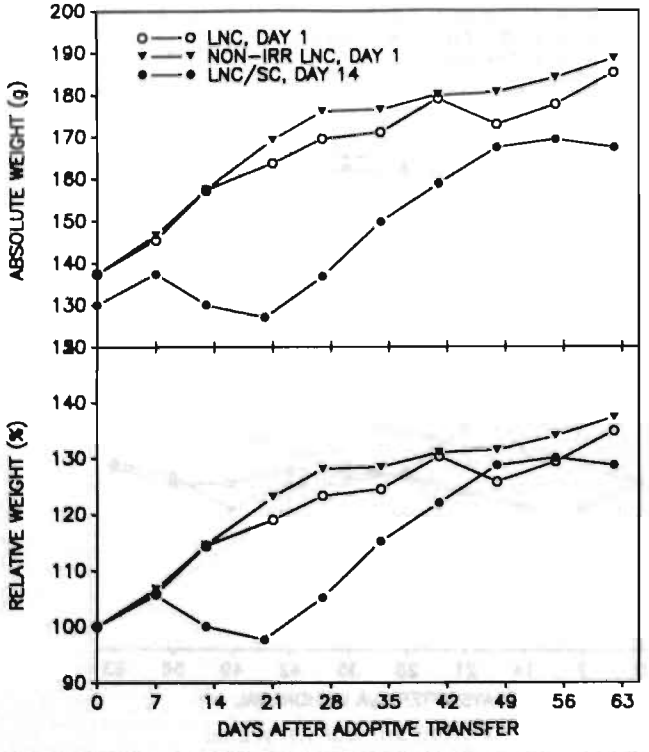

Figure 5.4: Effect of adoptive transfer of $3x10^7$ lymphoid cells obtained from rats treated for induction of CyA-AI on day 1 (o) and day 14 (e) after CyA withdrawal, on the weight of secondary recipients. Furthermore, the effect of transfer of lymph node cells obtained one day after withdrawal of CyA from rats only given CyA for 6 weeks, on the weight of secondary recipients is shown (▾). Upper panel, absolute weights; lower panel, relative weights.

obtained from 4 of these donors one day after CyA withdrawal elicited CyA-AI in 5 irradiated and reconstituted secondary recipients (Fig. 5.4).

Discussion

The data presented in this study corroborate the hypothesis put forward by others that in models of CyA-AI autoreactive cells are generated during CyA administration. Since CyA-AI is shown here to develop also if rats are thymectomized immediately after cessation of CyA administration, autoaggressive cells (or their inducers) already must reside extrathymically prior to CyA withdrawal.

The first arguments in favor of this hypothesis were provided by Cheney and Sprent, who demonstrated that adoptive transfer of spleen- and lymph node cells obtained from irradiated and reconstituted mice immediately after a 5-6 weeks course of CyA elicited acute, fatal CyA-AI in irradiated and reconstituted secondary recipients (Cheney and Sprent, 1985). In the present study, however, CyA-AI-associated pathology elicited by this protocol was not severe and did not result in death of the animals but after the acute phase evolved to mild, chronic skin pathology. Although the dose of CyA used in the study of Cheney and Sprent was somewhat higher than that applied by us (10.0 *versus* 7.5 mg/kg/day), the difference in severity of the pathology observed is more likely to be due to the different experimental animals used and/or differences in their microbiological status (Sorokin *et al.*, 1986).

A more striking difference, however, concerns the data from an experiment reported by Cheney and Sprent in which *non*-irradiated, CyA-treated mice were used as donors for adoptive transfer studies. Lymphoid cells obtained from these donors immediately after withdrawal of CyA also evoked severe, fatal CyA-AI upon transfer to irradiated, reconstituted mice (Cheney and Sprent, 1985). In the rat model, however, this experiment could not be reproduced. The possible reasons indicated above to explain the gradual differences in skin pathology observed using irradiated donors may also apply to the disconcordant findings regarding this protocol.

Both the results of our adoptive transfer studies using thymocytes, and the studies in which thymectomies were performed, indicate that neither at the time of CyA withdrawal, nor 2 weeks thereafter, the thymus represented an important reservoir of autoaggressive cells. On the other hand, transfer of 10^8 thymocytes collected from rats exhibiting CyA-AI 30 days after CyA withdrawal were shown to transfer CyA-AI to irradiated, reconstituted secondary recipients (Beschorner *et al.*, 1988a). We interpret this apparent discrepancy to reflect remigration of autoaggressive effector T cells to the thymus (Beschorner *et al.*, 1988b). Their putative destructive action may be the cause of the reduced cell yield from the thymus and secondary lymphoid organs after CyA withdrawal, as also noticed by others (Beschorner *et al.*, 1987a).

Thymectomy one day after withdrawal of CyA did neither have an effect on the kinetics of development, nor on the severity of skin pathology. On the other hand, weight loss occurred about two weeks later in Tx than in sham-Tx rats. This discrepancy suggests that the generation of the effector cells causing skin pathology may proceed differently from the generation of the effector cells which indirectly cause the weight loss in these animals. The former may already arrive *in situ* during the course of CyA, whereas the latter may still have to be generated and to home to the target organs after the decline of CyA levels in the rats.

Another model which yielded data pertinent to the subject of this study is that of transplantation of the thymus from CyA-treated *nu/+* mice to *nu/nu* recipients (Sakaguchi and Sakaguchi, 1988). In this model, autoimmunity manifested itself by autoantibody-induced gastritis, oophoritis, thyroiditis and/or insulitis 3 months after thymus transplantation. Thymus graft donors were either neonatal mice treated for 7 days with 10 mg/kg CyA or (non-irradiated) adult mice given 20 mg/kg CyA for 14 days. The data from this study suggested that the thymus may harbor inducers of CyA-AI early after the start of administration of CyA, whereas our results indicated that the (rat) thymus did not contain significant numbers of CyA-AI effector- or inducer cells after 6 weeks of CyA administration. Since the *nu/nu* recipients of CyA-treated thymus grafts were not given CyA, and CyA-induced changes in the thymus commence to reverse within a few days after CyA withdrawal (Beschorner *et al.*, 1987b), in the mouse thymus graft model a reservoir function of the thymus for autoreactive cells is more likely than *de novo* generation of such cells from precursors arriving from the recipient's bone marrow. Furthermore, phenotyping of thymocytes from irradiated mice treated with CyA for only 3 weeks demonstrated an arrest in the development of mature CD4 and CD8 single positive T lymphocytes and the occurrence of T cells expressing potentially autoreactive T cell receptors (Gao *et al.*, 1988; Jenkins *et al.*, 1988).

Taken together, our data and the studies referred to above suggest that the generation of autoreactive T cells may commence early after the start of CyA administration, but the continuous generation of these cells in the thymus under CyA administration is uncertain. As demonstrated in the present study, autoreactive cells leave the thymus during the course of CyA, but their targets for homing remain to be determined. The mild form of CyA-AI that developed after adoptive transfer of lymph node cells collected immediately after cessation of CyA administration suggested that autoreactive cells preferably home outside the secondary lymphoid organs (i.e. in the skin) or that inducer- rather than effector cells are exported from the thymus to the lymph nodes. In the latter case, expansion of autoaggressive clones (i.e. anti-MHC class II cytotoxic T cells (Hess *et al.*, 1985)) would not yet have occurred at the time of adoptive transfer. This expansion may not take place until about two weeks after CyA withdrawal when CyA concentrations have decreased to biologically ineffective levels (Bos *et al.*, 1988a). The discrepancy in the kinetics of weight loss and development of skin pathology in Tx rats as compared to sham-Tx rats suggests that both mechanisms may be operative.

Acknowledgements

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Chapter 6

Kinetics of inducer/effector cell generation in the thymus in Cyclosporine A - induced autoimmunity

Kinetics of inducer/effector cell generation in the thymus in Cyclosporine A - induced autoimmunity

Abstract

Lethally irradiated Lewis (LEW) rats reconstituted with syngeneic bone marrow and given Cyclosporine A (CyA) for 6 weeks following BMT develop, 2 weeks after cessation of CyA administration, a thymus-dependent autoimmune disease (Cyclosporine A-induced autoimmune disease; CyA-AI) resembling graft-versus-host disease (GVHD) after allogeneic BMT. The symptoms of disease include acute dermatitis and chronic scleroderma-like skin pathology. The role of the thymus in the etiology of CyA-AI has unequivocally been established. In the present study we investigated whether inducer/effector cells of CyA-AI are generated in the thymus and if so, at what time during CyA administration; whether these cells stay in the thymus.

Adoptive transfer of thymocytes obtained on different days after bone marrow transplantation from donors treated for induction of CyA-AI did not elicit CyA-AI in irradiated secondary recipients. Furthermore, thymectomy or sham-thymectomy of lethally irradiated, bone marrow reconstituted and CyA-treated rats after 12 (post-BMT) or more days of CyA therapy did elicit CyA-AI with development of skin pathology.

Therefore, this study demonstrates that CyA-AI inducer/effector cells are generated in the irradiated thymus within 12 days of CyA administration. These cells exit from the thymus within 12 days of CyA administration and reside elsewhere (i.e.. in the skin).

Introduction

Administration of Cyclosporine A (CyA) after *syngeneic* bone marrow transplantation (BMT) may induce autoimmune disease resembling the graft-versus-host disease (GVHD) secondary to *allogeneic* bone marrow transplantation (Glazier *et al.*, 1983a). This syngeneic GVHD (sGVHD)(Glazier *et al.*, 1983a), CyA-induced autoimmune disease (CyA-AI)(Sorokin *et al.*, 1986), or BMT-associated immune disease (BMT-ID)(Bos *et al.*, 1990) can be elicited reproducibly in young animals of certain rat (Fischer and Hess, 1990) or mouse (Cheney and Sprent, 1985; Bryson *et al.*, 1989) strains and has also been observed in adult humans (Jones *et al.*, 1989).

Several studies have demonstrated an essential role for the thymus in the induction of CyA-AI. Shielding of the thymus during total-body irradiation (TBI), as well as thymectomy prior to BMT, prevented the induction of CyA-AI (Glazier *et al.*, 1983b; Sorokin *et al.*, 1986) suggesting that the effector cells in CyA-AI are derived from the thymus. Rats thymectomized (Tx) 2, 4, and 6 weeks post-BMT developed CyA-AI disease, although somewhat delayed in terms of onset and time of death when compared to sham-Tx animals; suggesting a role for the thymus for several weeks after BMT (Sorokin *et al.*, 1986). At 6 weeks post-BMT these data were essentially confirmed by one of our own studies; furthermore we demonstrated in this study that at that time point and 14 days after CyA withdrawal CyA-AI could not be transferred by thymocytes. However, lymph node and spleen cells obtained from donors treated for induction of CyA-AI one and 14 days after cessation of CyA caused CyA-AI upon adoptive transfer to irradiated secondary recipients (Wodzig *et al.*, 1991; chapter 5). These studies, however, provided no explanation concerning the role of the thymus within 2 weeks post-BMT; especially at which stage during this fortnight after irradiation and BMT autoreactive T cells are generated in and released from the thymus under CyA administration. With respect to the kinetics of the thymic generation of autoreactive T cells in CyA-AI, it has been proposed that inducer and/or effector T lymphocytes are generated *during* CyA administration but remain inactive during CyA administration (Wilson, 1989). The disease becomes manifest only when CyA concentrations have declined to low levels. Manifestation of disease coincides with an increase of $CD4^+$ cells in the peripheral blood, suggesting these cells to be involved in the pathogenesis as inducer or effector cells (Bos *et al.*, 1988; Fischer *et al.*, 1991). At this time the disease can be transferred adoptively by lymph node $CD4^+$ T cells (Sorokin *et al.*, 1986) and both $CD4^+$ and $CD8^+$ splenic T cells (Fischer *et al.*, 1989; Hess *et al.*, 1990).

Studies on the transfer of CyA-AI with thymocytes suggested that at least part of the inducer/effector cells might remain in the thymus (Beschorner *et al.*, 1988a); after the decrease of the CyA concentrations in the hosts these autoreactive effector cells purportedly become active and destroy certain target cells (Majoor *et al.*, 1991). At which point in time putative autoreactive cells leave the thymus for the periphery is not clear nor has it been established unequivocally whether CyA might not also mediate an effect by interfering peripherally with tolerance induction.

The present study demonstrates that CyA-AI inducer/effector cells are generated within 12 days after BMT, most likely during the early phase of exponential thymocyte regeneration after irradiation. CyA-AI inducer/effector cells apparently leave the thymus (escape negative selection?) soon after their generation, and do not need the extrathymic presence of CyA in order to exert (auto-)reactive activity (Vanier and Prud'homme, 1992).

Materials and methods

Animals

Female, specific pathogen-free Lewis (LEW, RT1^{l}) rats were used as donors and recipients. Rats were obtained from our own breeding stock and were used at the age of 4-6 weeks.

Protocol for induction of CyA-AI

The experimental protocol has been described before (Bos *et al.*, 1988). In brief, rats were given 8.5 Gy at 0.5 Gy/min using a Röntgen irradiation machine (Philips MG320, Hamburg, Germany) one day prior to syngeneic BMT. Donor rats were killed by cervical dislocation under ether anesthesia. Bone marrow was collected from tibias and femurs of donor rats in Dulbecco's balanced salt solution supplemented with 2% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) (DBSS). Recipients received $6x10^7$ viable syngeneic bone marrow cells in 1.0 ml DBSS intravenously into a tail vein. CyA, a gift from Sandoz Co. Ltd., Basel, Switzerland, was dissolved in olive oil at a concentration of 7.5 mg/ml; rats received 7.5 mg/kg/day CyA subcutaneously for 4-42 days starting on the day of BMT.

Scoring of macroscopic pathology

After withdrawal of CyA, development of CyA-AI was assessed by examination of the rats at least twice a week for signs and symptoms of disease: red acra (hyperemia of the skin of the ears, feet and tail), inflammation of the eyelids, dermatitis, alopecia and weight loss (Wodzig *et al.*, 1991).

Thymectomy

Lewis rats treated according to the protocol for induction of CyA-AI were thymectomized (Tx) or sham-thymectomized (sham-Tx) on different days post-BMT when still treated with CyA. While under ketamine (Nimatek 50 mg/kg, intramuscularly) and xylazine (Sedamun 6 mg/kg, subcutaneously) anesthesia, rats were intubated and maintained on artificial respiration. The thorax was opened and all prepericardial soft tissue consistent with thymus was removed; the thorax and skin were closed using 4-0 suture. Rats under-

going sham-Tx were anesthetized, intubated, and an anterior mediastinectomy was performed. CyA administration was either stopped on the day of Tx or sham-Tx or continued up to day 42 days post-BMT.

Adoptive cell transfers

Thymocytes were obtained from thymectomy specimen. Single cell suspensions were made by passing thymic organ fragments through a 100 µm mesh width nylon gauze screen. Cells were collected in DBSS, washed, and their viability assessed by trypan blue exclusion. Hundred million viable thymocytes were administered either intravenously into a tail vein or intrathymically to 8.5 Gy irradiated and bone marrow-reconstituted secondary recipients. For intrathymic injection, rats were manipulated as for sham-Tx. The thorax was opened and fifty µl thymocyte suspension (concentration 10^9 thymocytes/ml) in DBSS was injected in each thymic lobe. To check for leakage from the thymic lobe 0.02% methylene blue was added to the thymocyte suspension.

Statistics

Differences with respect to incidence and severity of symptoms of CyA-AI were tested for statistical significance by Fischer's exact probability test.

Results

Recovery of thymocytes from CyA-AI rats

To investigate the appearance of autoreactive effector cells in the thymus during CyA administration, thymocytes obtained from irradiated and CyA-treated rats were transferred to irradiated and bone marrow-reconstituted secondary recipients. Thymocytes were obtained on day 4, 8, 12, 14, 16, 21, 28, and 35 after BMT from 5 donors per day. The mean yield of thymocytes from donor rats increased considerably between day 4 and day 8 after irradiation. On day 4, 2.5×10^6 thymocytes per rat were recovered, and on day 8, 3×10^8 thymocytes. Thereafter, mean thymocyte yields increased more slowly (day 12: 5×10^8; day 28 : 12.5×10^8) (Fig.6.1).

Failure to transfer CyA-AI with thymocytes

Thymocytes from donors taken on any day were pooled and transferred intravenously or intrathymically to 5 secondary recipients. At no day tested, however, adoptive transfer of 10^8 thymocytes, irrespective whether transferred intravenously or intrathymically to secondary recipients, elicited macroscopic symptoms of CyA-AI within 100 days.

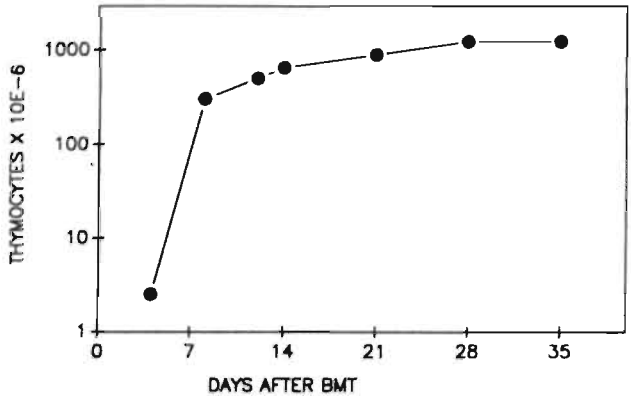

Figure 6.1: Recovery of thymocytes from CyA - AI rats. *Rats were treated for induction of CyA-AI and received CyA for 35 days. Each point represents the mean recovery from five rats.*

Effect of thymectomy and sham-thymectomy on development of CyA-AI

Irradiated and reconstituted rats were given CyA and underwent Tx or sham-Tx on day -1, 4, 8, 12, 14, 16, 21, 28, 35, or 42 post-BMT. On each given day groups of 10 rats were Tx; CyA was continued up to day 42 post-BMT in 5 rats and discontinued on the day of Tx in the remaining 5 rats. The latter protocol was also applied when sham-Tx rats were available. Rats thymectomized on day -1, 4, or 8 after BMT did not develop symptoms of CyA-AI for over 100 days, irrespective whether CyA was discontinued on the day of Tx or whether CyA was administered for 42 days (Table 6.1). Some rats thymectomized on day 12, 14, or 16 after BMT developed CyA-AI whereas other rats in these cohorts remained free of symptoms. In contrast, CyA-AI developed in almost all rats that underwent Tx or sham-Tx on days 21-42 post-BMT, irrespective of the duration of CyA treatment. These data show that from day 12 after BMT Tx no longer interferes with the development of CyA-AI. Neither did abrogation of CyA administration on the day of Tx or continuation up to 42 days after BMT influences the development of CyA-AI.

Discussion

Our data document the generation of CyA-AI inducer/effector cells in LEW rats as early as within 12 days after irradiation and syngeneic bone marrow transplantation. Since at no time point tested after BMT CyA-AI could be adoptively transferred using 10^8 thymocytes, the relative number of autoreactive T inducer/effector cells residing in the thymus must be quite low. This finding may be explained by assuming that autoreactive T cells exit from the thymus soon after their generation. It may be questioned, however,

Table 6.1: *Effect of thymectomy (Tx) on induction of CyA - AI[a].*

Treatment	Surgery performed on day post-BMT	N	Course of CyA after BMT (days)	Incidence and symptoms of CyA-AI[b]
Tx	-1	5	42	0/5
Sham-Tx		5	42	5/5 D (5), A (5)
None		5	42	5/5 D (5), A (5)
Tx	4	5	42	0/5
Tx		5	4	0/5
Tx	8	5	42	0/5
Tx		5	8	0/5
Tx	12	5	42	2/5 E (2), D (2)
Tx		5	12	2/5 E (2), D (2)
Sham-Tx		5	12	3/5 T (3)
Tx	14-16[c]	9	42	7/9 D (7), A (7)
Tx		9	14-16	6/9 E (1), D (5), A (5)
Sham-Tx		9	14-16	3/9 E (2), D (1), A (1)
Tx	21-42[d]	17	42	17/17 D (17), A (17)
Tx		19	21-42	17/19 D (17), A (17)

[a]All rats were X-irradiated on day -1 and received a BMT on day 0. [b]Symptoms developed 2-4 weeks after discontinuation of CyA; E, erythroderma; D, dermatitis; A, alopecia either ventral or generalized; T, thin fur (indicative of very mild disease). [c]Pooled data of day 14 and 16 after BMT. [d]Pooled data of day 21, 28, 35, and 42 after BMT.

whether transfer of 10^8 thymocytes would be enough to transfer CyA-AI. In another study (to be discussed below) following a different protocol, transfer of 10^8 thymocytes sufficed to elicit CyA-AI in secondary recipients (Beschorner *et al.*, 1988a). Furthermore, several studies have shown as few as 1-5x10^6 spleen- and lymph node cells to be adequate for transfer of disease from animals with CyA-AI to irradiated, and reconstituted secondary recipients (Sorokin *et al.*, 1986; Fischer *et al.*, 1991). Finally, usually about 10^9 thymocytes are harvested from the thymus of young LEW rats that recovered from irradiation and BMT. Therefore, 10^8 cells represent about 10% of all cells, making successful transfer of disease by larger amounts of thymocytes rather unlikely. Intrathymic introduction of thymocytes was attempted as an alternative route to see whether putative inducer/effector cells would need intrathymic processing in a CyA-free environment to become effective. However, the failure to transfer CyA-AI with thymocytes irrespective of the route of administration precludes any conclusion in this respect.

Apparently, autoreactive T cells are generated as unwanted by-products of the massive expansion of the number of thymocytes in the first 12 days of recovery after irradiation and reconstitution (a 200-fold increase between day 4 and 12 post-BMT or about 5x10^8 cells). Since especially early after BMT, and under CyA administration, intrathymic MHC class II expression is strongly suppressed, thymic negative selection mechanisms regularly preventing the release of harmful autoreactive T cells may not be operative (Boehmer von, 1986; Beschorner *et al.*, 1987, 1988b; Kisielow *et al.*, 1988). Although disease occurred after a regimen of CyA administration as short as 12-14 days after BMT, it manifested itself more frequently and more severely after prolongation of CyA administration for another week. The relative increase in thymocyte number in that period is only 2-fold, but the absolute accretion of about 5x10^8 thymocytes in that week may still contribute to the generation of autoreactive cells. Extending the period of CyA admin-

istration (even up to 12 weeks after BMT), however, has no clear effect on the incidence or severity of CyA-AI (Bos *et al.*, 1988). Recovery of thymic negative selection mechanisms or generation of counteractive cellular circuits from about 3 weeks post-BMT onwards are possible explanations for this observation.

Our results may appear to be in contrast to those reported by Beschorner *et al.* (1988a). Their study established the possibility to transfer CyA-AI to secondary recipients using either $8x10^7$ or $1.8x10^8$ thymocytes. In their study, however, thymocytes were harvested 30 days after discontinuation of CyA. At that time, dormant autoreactive cells have usually started to attack target structures. Possibly, migrating effector cells re-enter the thymus to destroy target cells, i.e. expressing MHC class II antigens (Hess *et al.*, 1985). Therefore, we explain the discrepancy with our present study by the presence of effector cells re-migrated to the thymus some weeks after discontinuation of CyA (Majoor *et al.*, 1991).

Another issue addressed in this study is whether the generation of CyA-AI effector cells is completed in the thymus, or whether extra-thymic effects of CyA contribute to effector cell generation. Rats thymectomized at days 12, 14-16 or 21-42 after BMT and given CyA for 42 days showed no statistically significant differences with respect to incidence and severity of symptoms of CyA-AI as compared to rats in which CyA was discontinued on the day of Tx. These results do not support a role for additional extrathymic processing of autoreactive T cells under CyA administration.

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Summary and general discussion

Cyclosporine A (CyA) is a potent immunosuppressive drug that is widely used clinically in the management of both alloimmune and autoimmune disorders. CyA, however, exerts a paradoxical effect on the immune system; it may also disturb the establishment of self-tolerance and induce autoimmune disease in animals.

In chapter 1, one form of CyA-induced autoimmunity which affects the thyroid, pancreas, gonads, adrenal gland, stomach, and salivary glands is described. In this model neonatal mice are given a 7-days course of CyA (Sakaguchi and Sakaguchi, 1989). Another form of autoimmunity may arise in lethally X-irradiated rats and mice of certain strains that are reconstituted with syngeneic bone marrow and treated daily with CyA for several weeks. After CyA is withdrawn, the vast majority of these rats develop a T cell-dependent autoimmune syndrome with pathology resembling in the acute phase that of graft-versus-host disease (GVHD) as seen after allogeneic bone marrow transplantation (BMT). In the chronic phase scleroderma-like lesions may develop. This syndrome has been referred to as Cyclosporine A-induced autoimmunity (CyA-AI)(Sorokin *et al.*, 1986) or syngeneic GVHD (Glazier *et al.*, 1983). A similar disease occurs in humans following withdrawal of CyA treatment in recipients of autologous or syngeneic bone marrow (Jones *et al.*, 1989; Hess *et al.*, 1992). It should be stressed that syngeneic GVHD has also been reported to occur incidentally in the absence of CyA therapy in both humans (Hood *et al.*, 1987) and experimental animals (Van Bekkum *et al.*, 1990). However, the occurrence of sGVHD is unpredictable in subjects that have not received CyA, and the disease may be mild and transient. Therefore, CyA treatment after lethal irradiation and bone marrow reconstitution provides a reproducible method of inducing a severe form of sGVHD.

Based on the fact that LEW rats are susceptible for several cell-mediated autoimmune diseases to which BN rats are resistant, and since LEW and (LEWxBN)F1 rats are susceptible for induction of CyA-AI, the aim of the study, as outlined in chapter 2, was to document whether susceptibility or resistance to CyA-AI is genetically a dominant trait. Susceptibility and resistance was established by macroscopic scoring of signs of disease like dermatitis, alopecia, weight loss, and histological examination of the skin. The resistance of the BN strain to the induction of CyA-AI *versus* the susceptibility of the LEW strain is demonstrated. Cell-mediated immune mechanisms were in part affected by CyA administration after X-irradiation and syngeneic bone marrow reconstitution. Delayed-type hypersensitivity (DTH) reactions were transiently strongly suppressed in the acute phase of CyA-AI in LEW rats but not so in CyA-AI resistant BN rats. On the other hand, immunohistochemical examination of the skin, with respect to cells involved in DTH reactions, and thymus of experimental animals revealed no differences between susceptible and resistant strains. Natural killer (NK) cell activity was also unaffected in both

strains. These findings are in agreement with the results from studies on the development of experimental allergic encephalomyelitis (EAE) in LEW rats treated with myelin basic protein (MBP), to which the BN strain is resistant (Mason, 1991). In contrast, BN rats are susceptible to some antibody-mediated autoimmune diseases (e.g. mercury chloride-induced glomerulonephritis) to which LEW rats are resistant. It has been shown recently that in the rat the $CD4^+$ T cells can be divided into two subpopulations based on the expression of cell surface markers CD45RC (as recognized by monoclonal antibody OX-22) and RT6. The $CD45RC^+RT6^-$ subset of rat $CD4^+$ T cells has a T helper 1 (T_H1)-like lymphokine repertoire (high IL-2 and IFNγ, but low IL-4 production) and provides cell-mediated immune responses, whereas the $CD45RC^-RT6^+$ population has characteristics of T helper 2 (T_H2)-type cells (IL-4 but low IL-2 and IFNγ production, provision of B cell help, and suppression of cell-mediated responses)(Fowell *et al.*, 1991; Fowell and Mason, 1993). In this context, it is interesting to note that normal BN rats (known to respond in a T_H2-like fashion) have a preponderance of the T_H2-like subset over T_H1, whereas for LEW rats (known to respond in a T_H1-like fashion) the opposite is the case (Groen *et al.*, 1993). The repertoire of cytokines produced can promote the development of either T_H1- or T_H2-type responses. Most significantly, IL-4, a product of T_H2-type T cells, inhibits the induction of T_H1-type responses, suggesting that a potent T_H2 reaction, as present in BN rats, is likely to decrease cell-mediated immunity (Fowell and Mason, 1993).

LEW rats subjected to the protocol for induction of CyA-AI initially showed symptoms akin to those seen in acute GVHD and in the chronic phase scleroderma-like lesions. Chapter 3 describes an experiment in which 77% of the experimental animals developed rapidly progressive subcutaneous and/or intracutaneous malignancies, notably sarcomas. These soft-tissue tumors developed late in the history of CyA-AI and appeared to be specifically associated with CyA-AI. No malignancies were observed in X-irradiated and syngeneic bone marrow-reconstituted or in CyA-treated control animals. How the tumors were generated is obscure, but we offer the following explanation. Our hypothesis is based on the observations that thymic inflammatory skin disease was present in virtually all animals with tumors, and that the tumors could be transplanted as explants or as a cell suspension to normal LEW rats. Furthermore, with respect to the collagen deposition chronic CyA-AI shows strong similarity to human (progressive) systemic sclerosis (SS) or scleroderma (Bos *et al.*, 1989) and several observations support a relationship between the development of cancer and the presence of SS (Medsger, 1985). Therefore, our hypothesis holds that these tumors were caused by a combination of X-irradiation and a chronic - cutaneous - inflammatory reaction and in this fashion are indirectly thymus-dependent.

Based on the similarities between SS and chronic GVHD, scleroderma or SS is believed to have an (auto)immune origin. Frequently, in patients with immune-mediated connective tissue diseases, and especially in SS, circulating autoantibodies against nuclear and/or cytoplasmic constituents were detected (Krieg and Meurer, 1988). Autoantibodies to DNA-topoisomerase I (Scl-86, Scl-70) and to centromere/kinetochore (CR19, CENP A-, B-, and C-antigens) were of prognostic and diagnostic importance for diffuse SS or limited scleroderma (Weiner *et al.*, 1988; Krieg and Meurer, 1988). Chapter 4 shows that

autoantibodies to extractable nuclear and cytoplasmic fractions are elicited in rats with CyA-AI. Furthermore, it was demonstrated that these autoantibodies are not required for, nor pathologically related to the development of scleroderma-like lesions. Finally, the specificity of some of the autoantibodies detected in LEW rats with CyA-AI disease also occurred in BN rats with mercury chloride-induced autoimmune nephritis, suggesting that analogous autoimmune mechanisms are involved.

Chapter 5 and 6 deal with the localization of effector cells and the kinetics of inducer/effector cell generation in the thymus in CyA-AI respectively. Several studies have demonstrated the pivotal role of the thymus in the etiology of CyA-AI. Shielding of the thymus during total body irradiation (Glazier *et al.*, 1983) or thymectomy prior to BMT prevented the induction of CyA-AI (Sorokin *et al.*, 1986). The onset of CyA-AI is associated with the reappearance of $CD4^+$T cells in the peripheral blood about two weeks after cessation of CyA administration (Bos *et al.*, 1988; Fischer *et al.*, 1991) and the development of $CD8^+$ cytotoxic T cells recognizing a public determinant of MHC class II antigen, preferentially the I-A subregion (RT1.B) of the MHC class II antigen complex (Hess *et al.*, 1985,1993). In chapter 5 the question is addressed whether inducer/effector cells of CyA-AI are generated in the thymus *during* or *after* CyA administration; whether these cells stay in the thymus and, if they don't, whether they home to the secondary lymphoid organs. Adoptive transfer of thymocytes from donor rats treated for induction of CyA-AI obtained one and 14 days after cessation of CyA administration did not elicit CyA-AI in secondary recipients. In contrast, studies by Beschorner *et al.*, (1988) on the transfer of CyA-AI with thymocytes harvested 30 days after discontinuation of CyA suggested that at least part of the inducer/effector cells might remain in the thymus. At that time, how-ever, autoreactive cells have usually already started to attack target structures. Probably, migrating effector cells re-enter the thymus to destroy target cells, i.e. expressing MHC class II antigens. Therefore, we explain the discrepancy between the study of Beschorner *et al.*, (1988) and our study by effector cells re-migrated to the thymus some weeks after discontinuation of CyA. Furthermore, adult thymectomy of rats immediately after the course of CyA did not influence the kinetics of development of skin pathology, although weight loss commenced later in thymectomized than in sham-thymectomized rats.

Lymph node and spleen cells obtained from donors treated for induction of CyA-AI one and 14 days after withdrawal of CyA caused CyA-AI upon adoptive transfer to secondary recipients, but the symptoms of acute disease were strikingly less severe upon transfer of lymphoid cells obtained one day after stopping CyA than 14 days thereafter. Therefore, chapter 5 demonstrates that CyA-AI inducer/effector cells are generated in the thymus *during* the administration of CyA. These cells exit from the thymus during CyA administration; either they home predominantly peripherally (i.e. into the skin) rather than in the secondary lymphoid organs, or they leave the thymus as inducer cells which home in the lymphoid organs where they subsequently may trigger potentially autoreactive lymphocytes as probably also present in normal individuals, or both pathways may be operative. Furthermore, there is considerable debate concerning the potency of T lymphocyte subsets from CyA-AI rats in transferring disease to secondary recipients.

Hess *et al,* (1990) demonstrated the requirement for both splenic $CD4^+$ and $CD8^+$ T lymphocytes (both 7.5 x 10^6) to transfer CyA-AI effectively. Transfer of large numbers (>3 x 10^7) of $CD8^+$ cells from animals with acute CyA-AI resulted in the induction of CyA-AI in the secondary recipients. However, lower numbers of $CD8^+$ cells could only transfer CyA-AI if accompanied by small numbers of $CD4^+$ cells from donors with CyA-AI. The $CD4^+$ subset cells harvested during acute CyA-AI were ineffective by themselves. On the other hand, Sorokin *et al.* (1986) were able to transfer disease with as few as 3 x 10^5 $CD4^+$ lymph node cells from animals with acute CyA-AI; B cells and $CD8^+$ cells were far less effective. It is of great importance to investigate in our setting whether the subset(s) of T lymphocytes causing CyA-AI are $CD4^+$ and/or $CD8^+$ T lymphocytes, and whether the same cells mediate the changes of chronic scleroderma observed in our model but not by others who have reported acute dermatitis only (Glazier *et al.*, 1983). In the rat, division of the T helper compartment into two functionally distinct subsets (see above) showed that the $CD4^+CD45RC^+$ helper subset was associated with lethal CyA-AI (and responsible for the production of IL-2), whereas the $CD4^+CD45RC^-$ helper subset appeared to be critical for effective autoregulation (Fischer *et al.*,1989; Hess *et al.*, 1990).

Chapter 6 describes that adoptive transfer of thymocytes obtained on different days after BMT from donors treated for induction of CyA-AI did not elicit CyA-AI in irradiated secondary recipients. Furthermore, thymectomy of lethally irradiated, bone marrow-reconstituted and CyA-treated rats after 12 or more days of CyA therapy post-BMT still permitted development of CyA-AI with skin pathology. Therefore, chapter 6 demonstrates that CyA-AI inducer/effector cells are generated in the irradiated thymus within 12 days of CyA administration, most likely during the early phase of exponential thymocyte regeneration after X-irradiation. These cells exit from the thymus (escape negative selection?) within these 12 days and migrate elsewhere (i.e. to the skin). These cells do not require the extrathymic presence of CyA in order to exert their (auto-)reactive activity. As demonstrated in this chapter, autoreactive cells leave the thymus during the course of CyA, but their targets for homing remain to be determined.

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Samenvatting en algemene discussie

Cyclosporine A (CyA) is een immunosuppressieve stof, die klinisch veel gebruikt wordt voor zowel het onderdrukken van transplantaat-afstoting als voor de behandeling van autoimmuunziekten. Het effect van CyA op het immuunsysteem is echter tegenstrijdig; het kan namelijk de tolerantie voor lichaams-eigen antigenen verstoren, hetgeen in dieren leidt tot de inductie van autoimmuunziekten.

In hoofdstuk 1 wordt een orgaan-specifieke vorm van CyA-geïnduceerde autoimmuniteit beschreven, waarbij de schildklier, alvleesklier, geslachtsorganen, bijnier, maag en speekselklieren worden aangedaan. In dit model wordt CyA gedurende 7 dagen aan pasgeboren muizen toegediend (Sakaguchi and Sakaguchi, 1989, 1992).
Een andere vorm van autoimmuniteit kan worden opgewekt na letale Röntgen bestraling van bepaalde ratte- en muizestammen, die worden gereconstitueerd met syngeen beenmerg en dagelijks gedurende enkele weken met CyA worden behandeld. Het merendeel van de ratten ontwikkelt een T-cel afhankelijk autoimmuun syndroom, nadat men de CyA-medicatie stopt, dat pathologisch gezien gelijkenis vertoont met de acute fase van een graft-versus-host ziekte (GVHD) na allogene beenmerg-transplantatie (BMT). In de chronische fase van de ziekte kunnen zich scleroderma-achtige afwijkingen ontwikkelen. Dit syndroom noemt men Cyclosporine A-geïnduceerde autoimmuniteit (CyA-AI) (Sorokin *et al.*, 1986) oftewel syngene GVHD (sGVHD)(Glazier *et al.*, 1983). In patiënten, die autoloog of syngeen beenmerg ontvingen, kan zich na het stoppen van de CyA-therapie een soortgelijke ziekte ontwikkelen (Jones *et al.*, 1989; Hess *et al.*, 1992). Het voorkomen van een syngene GVHD zonder CyA-therapie is zowel in de mens (Hood *et al.*, 1987) als in proefdieren (Van Bekkum *et al.*, 1990) zeer incidenteel en onvoorspelbaar, en de ziekte is mild en van voorbijgaande aard. Het toedienen van CyA aan immuun-gecompromitteerde individuen is dan ook een goede reproduceerbare methode om een ernstige vorm van syngene GVHD oftewel CyA-AI te induceren.

Het blijkt dat LEW ratten gevoelig zijn voor inductie van verscheidene cel-gemediëerde autoimmuunziekten waarvoor BN ratten resistent zijn. Aangezien LEW en (LEWxBN)F1 ratten gevoelig zijn voor inductie van CyA-AI, is het doel van hoofdstuk 2, te onderzoeken of induceerbaarheid of resistentie voor CyA-AI een genetisch dominante eigenschap is. Gevoeligheid en resistentie werden bepaald aan de hand van macroscopische score van ziekteverschijnselen zoals dermatitis, alopecia, gewichtsverlies en histologisch onderzoek van de huid. In dit hoofdstuk wordt de resistentie van de BN stam voor inductie van CyA-AI versus de gevoeligheid van de LEW stam aangetoond. Cel-gemediëerde immuunmechanismen worden ten dele door CyA-medicatie, na Röntgen bestraling en reconstitutie met syngeen beenmerg, aangetast. Vertraagd-type-overgevoeligheid (DTH) reakties worden in LEW ratten tijdelijk sterk onderdrukt in de akute fase van CyA-AI maar niet in CyA-AI resistente BN ratten. In de thymus oftwel zwezerik van experi-

mentele dieren vindt men immunohistochemisch geen verschil tussen gevoelige en resistente stammen tijdens en vroeg na de CyA-therapie. Tevens blijkt dat de huid van beide stammen voor wat betreft de cellen, noodzakelijk voor het ontstaan van dan wel betrokken zijn bij de DTH reaktie, geen verschillen vertoont. De NK-cel aktiviteit is in beide stammen onaangetast. Bovenstaande bevindingen zijn in overeenstemming met de resultaten verkregen uit andere studies. LEW ratten, die behandeld worden met het eiwit myeline ontwikkelen experimentele allergische encephalomyelitis (EAE). BN ratten zijn resistent voor de inductie van deze T-cel gemediëerde autoimmuunziekte (Mason, 1991). Het blijkt echter dat BN ratten gevoelig zijn voor inductie van antichaam-gemediëerde autoimmuunziekten zoals de door kwikchloride geïnduceerde ontsteking van de glomeruli.

Recentelijk is aangetoond dat de $CD4^+$ T cellen in de rat, op basis van expressie van de celoppervlakte markers CD45RC (herkend door monoclonaal antilichaam OX-22) en RT6, kunnen worden onderverdeeld in twee subpopulaties. De $CD45RC^+RT6^-$ subset van de ratten $CD4^+$ T cellen produceert een T helper 1 (T_H1)-achtig lymfokine repertoir (hoge IL-2 en IFNγ, maar lage IL-4 productie) en is betrokken bij cel-gemediëerde immuun responsen, terwijl de $CD45RC^-$ $RT6^+$ populatie kenmerken bezit van de T helper 2 (T_H2)-type cellen (hoge IL-4 maar lage IL-2 en IFNγ productie, ondersteunen van B cel hulp, en suppressie van cel-gemediëerde responsen)(Fowell *et al.*, 1991; Fowell and Mason, 1993). In relatie tot bovenstaande is het interessant op te merken dat bij normale BN ratten (reagerend op een T_H2-achtige manier) de T_H2/T_H1-ratio sterk in het voordeel van de T_H2 subset is, terwijl dit voor LEW ratten (reagerend op een T_H1-achtige manier) veel minder geldt (Groen *et al.*, 1993). Afhankelijk van het geproduceerde lymfokine repertoir kan de ontwikkeling van T_H1- of juist T_H2-type responsen gestimuleerd worden. Zo kan IL-4, een produkt van T_H2-type T cellen, de inductie van T_H1-type responsen remmen. Dit suggereert dat een sterke T_H2 reaktie, zoals aanwezig bij BN ratten, de cel-gemediëerde immuniteit verlaagt (Fowell and Mason, 1993).

LEW ratten die onderworpen worden aan het protocol voor inductie van CyA-AI vertonen in beginsel symptomen identiek aan akute graft-versus-host ziekte (GVHD) en in de chronische fase scleroderma-achtige afwijkingen. Hoofdstuk 3 beschrijft een experiment waarin 77% van de experimentele dieren snel progressieve onderhuidse maligniteiten (neoplásmata) ontwikkelen, met name sarcomen. Deze weke-delen tumoren ontwikkelen zich laat in het verloop van CyA-AI en lijken specifiek geassocieerd te zijn met CyA-AI. Er worden geen maligniteiten gezien na Röntgen bestraling en reconstitutie met syngeen beenmerg of in CyA-behandelde controle ratten. Het is onduidelijk hoe de tumoren veroorzaakt worden maar we poneren de volgende hypothese welke is gebaseerd op de observatie dat bijna alle ratten met tumoren ook thymus-afhankelijke huidontstekingen hadden. De tumoren konden als explant of celsuspensie naar normale LEW ratten worden getransplanteerd. Bovendien blijkt in de rat, dat met betrekking tot de collageen depositie, CyA-AI sterke overeenkomsten vertoont met (progressieve) systemische sclerosis (SS) bij de mens (Bos *et al.*, 1989). Verscheidene observaties ondersteunen een relatie tussen de ontwikkeling van maligniteiten en de aanwezigheid van SS (Medsger, 1985). Concluderend zegt onze hypothese dat deze tumoren worden veroorzaakt door een

combinatie van Röntgen bestraling en chronische - huid - ontstekingsreakties en op deze wijze dus indirekt thymus-afhankelijk zijn.

Op basis van overeenkomsten tussen SS en chronische GVHD wordt verondersteld dat sclerodermie of SS veroorzaakt wordt door een (auto)immuun-reaktie. Bovendien blijkt dat patiënten met immuun-gemediëerde bindweefselziekten, en vooral SS, circulerende autoantilichamen hebben tegen kern- en/of cytoplasmatische macromoleculen (Krieg and Meurer, 1988). Autoantilichamen tegen DNA-topoisomerase I (Scl-86, Scl-70) en tegen de centromeer/kinetochoor (CR19, CENP A-, B-, en C-antigenen) zijn van prognostische en diagnostische waarde voor diffuse SS of gelimiteerde sclerodermie (Weiner *et al.*, 1988; Krieg and Meurer, 1988). Hoofdstuk 4 beschrijft dat auto-antilichamen tegen extraheerbare kern- en cytoplasmatische fracties worden opgewekt in ratten met CyA-AI. Verder wordt gedemonstreerd dat deze autoantilichamen niet noodzakelijk zijn voor, noch pathologisch gerelateerd zijn aan de ontwikkeling van de scleroderma-achtige afwijkingen. Tenslotte blijkt dat de specificiteit van enkele van deze autoantilichamen in LEW ratten met CyA-AI ziekte ook voorkomt in BN ratten met kwikchloride-geïnduceerde autoimmuun ontstekingen van de glomeruli, hetgeen suggereert dat analoge autoimmuun-mechanismen hierbij betrokken zijn.

De hoofdstukken 5 en 6 beschrijven enerzijds de localisatie van effector cellen en anderzijds de kinetiek van het genereren van inducer/effector cellen in de thymus bij CyA-AI. Verscheidene studies hebben de centrale rol van de thymus in de etiologie van CyA-AI beschreven. Afscherming van de thymus tijdens de totale lichaamsbestraling (Glazier *et al.*, 1983) of verwijdering van de thymus voor de beenmergtransplantatie voorkomt de inductie van CyA-AI (Sorokin *et al.*, 1986). Het begin van CyA-AI is enerzijds geassocieerd met het terugkeren van $CD4^+$ T cellen, ongeveer twee weken na het beëindigen van de CyA-behandeling, in het perifere bloed (Bos *et al.*, 1988; Fischer *et al.*, 1991) en anderzijds met de ontwikkeling van $CD8^+$ cytotoxische T cellen die een gemeenschappelijke determinant van het MHC klasse II antigeen herkennen (Hess *et al.*, 1985). In hoofdstuk 5 komt de volgende vraag aan de orde: worden inducer/effector cellen in CyA-AI tijdens of na het beëindigen van de CyA behandeling in de thymus gegenereerd; blijven deze cellen in de thymus en, zo niet, gaan ze preferentiëel naar de secundaire lymfoide organen ? Transfer experimenten met thymocyten, geïsoleerd 1 en 14 dagen na het stoppen van de CyA-medicatie uit donor ratten behandeld voor inductie van CyA-AI, wekken geen CyA-AI op in bestraalde ontvanger ratten.

In tegenstelling tot onze resultaten suggereren de studies van Beschorner *et al.*, (1988), waarin CyA-AI wordt overgebracht met behulp van thymocyten die 30 dagen na stoppen van de CyA-behandeling werden geïsoleerd, dat tenminste een deel van de inducer/effector cellen in de thymus aanwezig blijven. Op dit tijdstip zijn autoreaktieve cellen in de regel echter al begonnen met het aanvallen van hun doelwit. Het is waarschijnlijk dat migrerende effector cellen de thymus opnieuw binnen dringen en de doelwit cellen, die MHC klasse II antigenen tot expressie brengen, vernietigen. Daarom verklaren we het verschil tussen de studie van Beschorner *et al.*, (1988) en onze resultaten door effector cellen, die enkele weken na de beëindiging van de CyA-behandeling naar de thymus terug migreren. Bovendien blijkt een thymectomie meteen na de kuur van CyA bij volwassen

ratten de kinetiek van de ontwikkeling van huid pathologie niet te beïnvloeden, hoewel gewichtsverlies in gethymectomeerde ratten later plaats vindt dan in schijn-gethymectomeerde dieren. Transfer van lymfeklier- en miltcellen, geïsoleerd 1 en 14 dagen na het stoppen van de CyA-medicatie uit donor ratten behandeld voor inductie van CyA-AI, wekken CyA-AI op in bestraalde ontvanger ratten. Echter de symptomen van akute ziekte bij de ontvanger ratten zijn veel minder ernstig bij transfer van lymfoide cellen die 1 dag na het stoppen van CyA uit donor ratten werden verkregen in vergelijking met cellen verkregen 14 dagen na het beëindigen van de CyA-behandeling. Hoofdstuk 5 demonstreert dat CyA-AI inducer/effector cellen gedurende de CyA-medicatie in de thymus worden gegenereerd. Deze cellen verlaten de thymus gedurende de CyA-behandeling om vervolgens enerzijds bij voorkeur naar perifere organen (bijvoorbeeld de huid) te gaan i.p.v. naar de secundaire lymfoide organen of anderzijds verlaten ze de thymus als inducer cellen, die naar de lymfoide organen toe gaan en mogelijkerwijs potentieel autoreaktieve cellen, die ook in normale individuen aanwezig zijn, aanzetten. Het is echter ook mogelijk dat een combinatie van beide mechanismen van toepassing is. Er bestaat onenigheid omtrent de potentie van T-lymfocyt subsets om CyA-AI naar secundair bestraalde ontvanger ratten te transfereren. Hess *et al.* (1990) heeft aangetoond, dat zowel $CD4^+$ als $CD8^+$ T lymfocyten (beide 7.5 x 10^6) verkregen uit de milt, vereist zijn om CyA-AI effectief over te brengen. Transfer van hoge aantallen (>3 x 10^7) $CD8^+$ cellen verkregen uit dieren met akute CyA-AI leidt tot inductie van CyA-AI in de bestraalde ontvangers. Lage aantallen $CD8^+$ cellen kunnen CyA-AI echter alleen over brengen samen met lage aantallen $CD4^+$ cellen uit donoren met CyA-AI. De $CD4^+$ cellen, die geïsoleerd werden tijdens akute CyA-AI, zijn zelf niet in staat om CyA-AI over te brengen. Sorokin *et al.* (1986) was echter wel in staat om met slechts 3 x 10^5 $CD4^+$ lymfeklier cellen, uit dieren met akute CyA-AI, de ziekte te transfereren; B cellen en $CD8^+$ cellen zijn veel minder effectief. Het is dan ook belangrijk om in ons model te onderzoeken of de T lymfocyt subsets, die CyA-AI veroorzaken, $CD4^+$ en/of $CD8^+$ lymfocyten zijn en of deze cellen tevens een rol spelen bij de veranderingen tijdens chronische scleroderma, terwijl andere studies alleen akute dermatitis constateren (Glazier *et al.*, 1983). Verdeling van het T helper compartiment in de rat in twee functioneel verschillende subsets (zie boven) suggereert dat de $CD4^+CD45RC^+$ helper subset geassocieerd is met letale CyA-AI (en verantwoordelijk voor IL-2 produktie), terwijl de $CD4^+CD45RC^-$ helper subset kritisch lijkt voor de effectieve regulatie van de ziekte (Fischer *et al.*, 1989; Hess *et al.*, 1990).

Hoofdstuk 6 beschrijft dat transfer experimenten van thymocyten, geïsoleerd op verschillende dagen na beenmergtransplantatie uit donoren behandeld voor inductie van CyA-AI, niet in staat zijn CyA-AI op te wekken in bestraalde ontvangers. Bovendien blijkt dat bestraalde, beenmerg gereconstitueerde en CyA-behandelde ratten, die gethymectomeerd worden na 12 of meer dagen van CyA-therapie na BMT, CyA-AI met huid-pathologie ontwikkelen. In dit hoofdstuk wordt aangetoond dat CyA-AI inducer/effector cellen in de bestraalde en CyA-behandelde thymus binnen 12 dagen, voornamelijk tijdens de vroege fase van exponentiële regeneratie van thymocyten na Röntgen bestraling, worden gegenereerd. Deze cellen verlaten de thymus (en ontsnappen aan negatieve selectie?) binnen 12 dagen en migreren naar elders (bijvoorbeeld de huid). Bovendien blijkt dat deze cellen om hun (auto-)reaktiviteit uit te oefenen geen extrathymale aanwezigheid van CyA vereisen.

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Curriculum vitae

Will Wodzig werd op 5 februari 1963 geboren in Kerkrade. In 1981 werd, na het behalen van het Atheneum-ß diploma aan het St. Antonius Doctor College te Kerkrade, begonnen met de studie Biologie aan de Katholieke Universiteit Nijmegen. In 1982 en 1984 respectievelijk werd het propedeutisch en kandidaats examen Biologie (B4) met goed gevolg afgelegd. De doctoraalstudie omvatte het hoofdvak Biochemie (Dr. W.J. van Venrooij; Dr. W.J. Habets) en de bijvakken Cytologie (Dr. A.M. Pieck) en Immunologie (Prof.Dr. P.J.C. van Breda Vriesman, vakgroep Immunologie, Rijksuniversiteit Limburg, Maastricht). Het doctoraal examen Biologie werd in november 1988 behaald.

Van 1 januari 1989 tot 31 december 1992 is hij als wetenschappelijk onderzoeker met financiele steun van het Reumafonds werkzaam geweest bij de vakgroep Immunologie van de Rijksuniversiteit Limburg. Onder leiding van Prof.Dr. P.J.C. van Breda Vriesman en Dr. G.D. Majoor werd gedurende deze periode het onderzoek verricht beschreven in dit proefschrift. Tijdens de promotieperiode is het diploma Proefdierdeskundigheid behaald.